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To the Graduate Council:

I am submitting herewith a thesis written by Megan Gray MacDougal entitled "Adipose Tissue's Potential Role as a Reproductive or Lactation Endocrine Gland." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

J. Lannett Edwards, Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:
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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Adipose Tissue's Potential Role as a Reproductive or Lactation Endocrine Gland

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Megan Gray MacDougal

December 2012

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ABSTRACT

While adipose tissue secretes hormones related to nutrition and metabolism, a few studies have provided evidence suggesting a direct reproductive role from adipose-derived products. The goal of this study was to determine if adipose tissue serves as a reproductive or lactation endocrine gland. Adipose tissue was associated with the reproductive tract of mature female cows in two locations, mesosalpinx and mesometrium (tissues supporting the oviduct and uterus, respectively), in varying amounts. Using quantitative polymerase chain reaction on a non-descript pool of cows, LHβ, CGA, PRL, FST, and LEP transcripts were demonstrated to be present in mesosalpinx, subcutaneous, visceral, and peri-renal adipose depots. Subsequent efforts aimed to determine the influence of adiposity on transcript abundance. Body condition score was used to separate cows into two adiposity groups, which were confirmed by adipocyte size. Adiposity did not influence the abundance of LHβ, but the CGA transcript was influenced by the adiposity by depot interaction. The PRL transcript was also not influenced by adiposity. The FST transcript was more abundant in BCS 3 cows, while LEP was more abundant in BCS 6 cows. Protein efforts utilizing an RIA revealed the presence of luteinizing hormone in all four adipose depots. Using an antibody against purified pituitary-derived prolactin, a band was detected in 6/9 mesosalpinx adipose depots similar in size to that observed in the pituitary. Presence of transcripts and hormones in adipose tissue provides the framework necessary for adipose to be a reproductive or lactation endocrine gland, though secretion would also be required. Secretion of such hormones by specific adipose depots could provide local effects to nearby tissues.

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CHAPTER 1

INTRODUCTION

Obesity is a worldwide problem that continues to increase in incidence. Associated diseases include diabetes mellitus, cardiovascular disease, and osteoarthritis (reviewed by Norman and Clark, 1998), costing the United States over $100 billion per year (Finkelstein et al., 2009; Tsai et al., 2011). Along with contributing to many health risks, obesity negatively affects fertility. The odds for pregnancy in women decline by 0.84 for every unit increase in body mass index (Ferlitsch et al., 2004). Obesity also increases the rate of abortions and miscarriages in women (Cnattingius et al., 1998; Wang et al., 2002; Bellver et al., 2003; Cedergren, 2004; Lashen et al., 2004). Obesity negatively affects fertility in other species such as mice (Jungheim et al., 2010), chickens (Robinson and Wilson, 1996; Chen et al., 2006) and cattle (Morrow, 1976; Houghton et al., 1990; Leroy et al., 2010).

The problems associated with the obese state, including infertility, may be due to the role adipose tissue plays as an endocrine gland. While adipose tissue is most commonly known to store (Ahima, 2006) and release energy when the body requires it (Kim and Moustaid-Moussa, 2000), it also functions to secrete adipokines (Ahima, 2006). Adipokines, which are hormones, cytokines, or peptides secreted by adipose tissue, help the body maintain proper energy balance (Bohler et al., 2010) and regulate metabolism and food intake (reviewed by Mitchell et al., 2005). Many adipokines have receptors located in reproductive tissues (reviewed by Mitchell et al., 2005), such as leptin receptors in the ovary (Karlsson et al., 1997), and can have reproductive effects. For example, leptin administration in follicle stimulated hormone (FSH)-primed rats

caused a significant decrease in ovulation compared to those receiving saline (Duggal et al., 2002).

Serendipitous findings from microarray studies of adipose tissue provide some evidence of a possible reproductive endocrine role. Transcripts for luteinizing hormone beta-subunit (LH*β*) and follicle stimulating hormone beta-subunit (FSHβ) were detected in omental adipose of a middle-aged woman (Yang et al., 2003b). In addition to these transcripts, Barb et al. (2005) also found the glycoprotein hormone alpha-chain (CGA) transcript in pig adipose, while Hausman et al. (2006, 2008) also found follistatin (FST) transcript in subcutaneous adipose of neonatal and young pigs.

The working hypothesis of this thesis is that bovine adipose tissue associated with the reproductive tissues expresses the transcripts for and secretes reproductive hormones that could, in turn, be directly affecting reproductive functions. To test this hypothesis, the overall objective was to determine the extent to which adipose tissue in the female bovine produces reproductive hormones. As a first step, reproductive tracts from mature females were examined for adipose tissue. After establishing that adipose tissue was present on reproductive tracts, a next step was to determine the presence of reproductive hormone transcripts in mesosalpinx-associated adipose tissue from a pool of non-descript mature cows. Adipose tissue from different locations may function differently, evidenced by different mRNA expression levels of leptin, insulin receptor, PPAR-γ, GLUT4 (Lefebvre et al., 1998), and angiotensinogen (Dusserre et al., 2000) between subcutaneous and visceral depots. Therefore, it was important to examine adipose from other depots in addition to that associated with the reproductive tract. Depots of interest included subcutaneous, visceral, and peri-renal.

Next efforts investigated adipose tissue from mature cows of varying adiposity because it has been reported that large adipocytes function differently than small adipocytes. Skurk et al. (2007) demonstrated increased secretion of leptin, IL-6, IL-8, TNF-α, adiponectin, and other chemokines from very large adipocytes compared to very small adipocytes within an individual. Animals utilized in this effort were selected for homogeneous breed and reproductive status. Histological studies of the adipose tissue found in the mesosalpinx and subcutaneous depots were analyzed for adipocyte size because size increases with adiposity (Hirsch and Batchelor, 1976; Spalding et al., 2008). Transcripts of interest were investigated in the four previously mentioned adipose depots. Luteinizing hormone (LH), prolactin, and growth hormone proteins were investigated in order to determine if these transcripts were being translated in adipose tissue. Blood samples were also evaluated to determine the reproductive and metabolic/nutritional status of the cows. Additional efforts were made to determine if transcripts of interest in mesosalpinx adipose were located in the adipose itself, or the surrounding cells originating from the broad ligament.

CHAPTER 2

REVIEW OF LITERATURE

Introduction

In an attempt to discover a possible new reproductive role for adipose tissue secreting reproductive hormones, the following is a review of literature highlighting the risks and costs associated with obesity, obesity's effect on reproduction, and adipose tissue's role as an endocrine organ. Furthermore, the possibility for adipose to produce reproductive hormones and consequences of over secretion of reproductive hormones will be examined. Finally, systemic levels of reproductive hormones seen in obese individuals, the association of adipose tissue with the reproductive tract, and possible routes for hormone transport in reproductive tissues will be discussed.

Risks and Costs Associated with Obesity

Obesity, a condition characterized by an excessive accumulation of adipose tissue, is becoming a very serious problem in the world today. A person of normal weight is classified as having a body mass index (BMI) of 18.5 to 25, while an obese person has a BMI over 30 (Finkelstein et al., 2009). Obesity's prevalence is greatest in developed and developing countries, where access to high-fat food and a sedentary lifestyle are becoming more common (reviewed by Norman and Clark, 1998). There are a number of diseases associated with obesity: type II diabetes mellitus, osteoarthritis, cardiovascular disease, and sleep apnea (reviewed by Norman and Clark, 1998). The risk factors and diseases associated with obesity are detrimental to the individuals affected, thus creating emotional strains and physical suffering. In addition to emotional and physical impacts, obesity also produces an extreme economic consequence. In the

United States alone, overweight and obesity-related healthcare costs are approximately $100 billion per year (Tsai et al., 2011) and result mostly from costs required to treat the associated diseases (Finkelstein et al., 2009). Finkelstein et al. (2009) estimated that the medical spending for an obese individual is $1,429 more per year than someone of normal weight.

Obesity's Effects on Reproduction

Along with its contribution to many health risks and healthcare costs, obesity negatively affects the reproductive system of multiple species. One general reproductive effect many obese women experience is menstrual abnormality; such as oligomenorrhea (abnormally long or absent menstrual cycles), oligohypermenorrhea (heavy and irregular menstruation), oligohypomenorrhea (very light and irregular menstruation), and polymenorrhea (frequent menstruation; Hartz et al., 1979). In terms of fertility, pregnancy rates in women decline by 0.84 for every unit increase in BMI (Ferlitsch et al., 2004). Obese women are also more likely to have abortions and miscarriages as compared to normal weight women (Cnattingius et al., 1998; Wang et al., 2002; Bellver et al., 2003; Cedergren, 2004; Lashen et al., 2004). That many obese women have undergone in vitro fertilization has led to some possible mechanisms for the reproductive dysfunction caused by obesity. The percentage of good quality oocytes decreases in overweight women compared to normal weight women (Wittemer et al., 2000). In a widely used mouse model for human obesity, oocyte maturation takes longer in diet-induced obese mice (Jungheim et al., 2010). Over-conditioned cows, those having a fat body condition, experience decreased pregnancy rates, decreased first service conception rates (Houghton et al., 1990), retained fetal membranes

(Morrow, 1976), and decreased embryonic cell number (Leroy et al., 2010). Pig breeds with an obese phenotype, when compared to lean breeds, have reduced fertility, and fetuses experience intrauterine growth retardation (Gonzalez-Añover et al., 2011).

Obesity in males also results in reproductive dysfunction, though the effects in obese males are less evident than in females. Obesity has been demonstrated to alter spermatogenesis, resulting in a reduction in sperm count (reviewed by Hammoud et al., 2006). Increasing BMI in men is typically inversely related to normal motile sperm (Kort et al., 2006; Hammoud et al., 2008a; Hammoud et al., 2008b), sperm concentration (Hammoud et al., 2008a; Hammoud et al., 2008b; Bakos et al., 2011b), and total sperm count (reviewed by Hammoud et al., 2006; Hammoud et al., 2008a; Stewart et al., 2009). Sperm concentrations were observed to be decreased in obese mice when compared to lean (Bakos et al., 2011b) and sperm from obese mice have decreased fertilization rates (Bakos et al., 2011a). Another factor which contributes to obese male fertility problems is an elevated scrotal temperature. Excessive adipose in the scrotum and surrounding areas can have an insulating effect, which further exacerbates problems in sperm parameters (reviewed by Du Plessis et al., 2010)

Obesity not only directly impacts males, but can also have a negative impact on their offspring. Embryos derived from sperm of obese mice have reduced cleavage rates, delayed development, and are less likely to reach the blastocyst stage compared to lean counterparts (Mitchell et al., 2011). Resultant blastocysts from obese males have decreased cell numbers and implantation rates (Mitchell et al., 2011). It was recently reported that diet-induced obesity in male mice leads to reduced sperm function in their sons and grandsons as well as reduced meiotic competence of oocytes

in their daughters (Fullston et al., 2012). Similar results have been reported in humans as pregnancy rates, implantation rates, and live birth rates in pregnancies created using artificial reproductive technologies decrease as paternal BMI increased (Bakos et al., 2011b). One possible explanation may be the altered endocrine environment found in obese males. In men, this environment is characterized by decreased testosterone and gonadotropin (follicle-stimulating hormone and luteinizing hormone) levels, as well as increased estrogen levels (reviewed by Hammoud et al., 2006). Although the mechanisms for these reproductive dysfunctions have not been identified, adipose tissue and its role as an endocrine gland could be one of the causative factors.

Adipose Tissue as an Endocrine Organ

While adipose mainly functions to store (Ahima, 2006) and release energy when the body requires it (Kim and Moustaid-Moussa, 2000), it also functions as an endocrine gland through its secretion of hormones (Ahima, 2006). As well as providing energy directly, adipose tissue secretes adipokines to help the body maintain proper energy balance (reviewed by Bohler et al., 2010) and regulate metabolism and food intake (reviewed by Mitchell et al., 2005). Adipokines can be hormones, cytokines, or anti-inflammatory factors which work to regulate many systems within the body (reviewed by Campos et al., 2008). Some of the better-characterized adipokines include leptin and adiponectin. Leptin signals the body on its energy status (reviewed by Bohler et al., 2010). For example, leptin levels decrease shortly after beginning a fasting period (Boden et al., 1996) and increase with obesity in both circulation and in tissues (reviewed by Mitchell et al., 2005). Unlike leptin, adiponectin levels decrease with obesity. Adiponectin is an insulin-sensitizing agent that acts in reducing fatty acid

oxidation in the liver as well as reducing the activity of some gluconeogenic enzymes (reviewed by Mitchell et al., 2005).

Two less-commonly known adipokines are resistin and visfatin. Resistin levels in circulation have not been definitively related to obesity because levels vary between studies (reviewed by Mitchell et al., 2005). Resistin's role, while currently unclear, seems to vary between species and might relate to insulin resistance (reviewed by Bohler et al., 2010). Visfatin has insulin-like properties (reviewed by Bohler et al., 2010) such as lowering blood glucose and binding to insulin receptors (reviewed by Campos et al., 2008) and levels are directly proportional to the amount of visceral adipose in both mice and humans (reviewed by Bohler et al., 2010).

Adipose tissue acting as an endocrine gland may explain the negative effects of obesity on fertility. Many adipokines have receptors located in reproductive tissues (reviewed by Mitchell et al., 2005) and some are actually expressed within reproductive structures. For example, leptin and adiponectin expression was found in similar patterns in the rat ovary and oviduct (Archanco et al., 2007). Leptin stimulates gonadotropin secretion and steroidogenesis, events which are crucial to reproductive function (reviewed by Campos et al., 2008). Leptin receptor expression has been reported in the bovine anterior pituitary and leptin can stimulate the secretion of LH by acting directly at this location (Amstalden et al., 2003). Leptin receptor mRNA has also been detected in bovine corpus luteum, granulosa, and theca internal cells, providing a means to affect steroidogenesis in these locations (Sarkar et al., 2010). Some adipokines have also been reported to decrease fertility. For example, leptin

administration in FSH-primed rats caused a significant decrease in ovulation compared to those receiving saline (Duggal et al., 2002).

In addition to regulating energy balance, adipose secretes adipokines with a variety of other roles. The function of these secreted products can vary between the different adipose depots in the body (reviewed by Wozniak et al., 2009). For example, interleukin-6 and tumor necrosis factor alpha from epicardial adipose affect local inflammation and chemotaxis, while from muscular adipose affect insulin resistance (reviewed by Wozniak et al., 2009). Adipose tissue is also known to take up and interconvert sex steroid hormones (Deslypere et al., 1985). Due to the presence of aromatase within adipose tissue, androgens are easily converted to estrogens (Boulton et al., 1992). This enzymatic activity could explain the sex steroid hormone imbalance observed in the obese state. For instance, circulating testosterone levels in men are negatively correlated with BMI, while estrone levels are positively correlated (Bélanger et al., 2006).

Possibility for Adipose to Produce Reproductive Hormones

The most well-known functions of adipose tissue are related to metabolism and energy balance, however, adipose may have more roles to be elucidated. The working hypothesis of this thesis is that adipose tissue may be producing reproductive hormones and may have a direct impact on fertility. This hypothesis is based on the fertility issues noted in the obese state as well as the following literature supporting adipose tissue's possible reproductive endocrine role. A few studies have reported the presence of transcripts for reproductive hormones in adipose tissue, typically from microarray data

(Table 1). Given that the objectives of these studies focused on non-reproductive hormone transcripts, thus many were not followed up with validation experiments. Of particular interest is a study on neonatal pig adipose tissue which demonstrated novel expression of follicle stimulating hormone beta-subunit (FSH-β), luteinizing hormone beta-subunit (LH-β), and glycoprotein hormone alpha chain (CGA) (reviewed by Barb et al., 2005). These subunits combine to make FSH and LH, the gonadotropins responsible for follicular growth and initiating ovulation, respectively (reviewed by Gloaguen et al., 2011; Luo et al., 2011).

Similar to their findings published in the review paper, Hausman et al. (2006) found FSH-β, LH-β, and follistatin expressed in subcutaneous adipose tissue and/or stromal-vascular cell culture (not specified) of neonatal and fetal pigs. Follistatin was identified in adipose tissue by MALDI-TOF analysis and also gene microarray or protein array studies (not specified). This demonstrated that adipose not only contained the follistatin transcript, but translated it into protein. These findings were not discussed in the context of their possible relevance to reproduction, but were simply classified as novel or unusual gene expression. A follow-up study in 2008 by Hausman et. al reported the follistatin transcript in middle subcutaneous adipose tissue of young pigs (90-210 days) by RT-PCR in addition to microarray. These findings were particularly interesting to us due to the evidence supporting a possible role adipose tissue may have as a reproductive endocrine gland.

There have been a few studies on human adipose tissue whose findings parallel those seen in the pig. Yang et al. (2003) studied abdominal omental adipose from a 59-year-old non-obese woman using cDNA microarray from Expressed Sequence Tags

Table 1: Is adipose a reproductive endocrine gland?

Reference	Adipose Location	Species	Age	Method or Procedural Analysis	Transcriptomes of Reproductive Hormones Present
Barb et al., 2005	Not stated	Pig	Not stated	Microarray analysis	FSH-β[2] LH-β[3] CGA[6]
		Goat	Not Stated	Proteomic analysis of cell culture media	Prolactin
Hausman et al., 2006	Subcutaneous	Pig	Neonatal and fetal (90 and 105 d gestation)	cDNA microarray from 560 pig gene sequences	FSH-β[2] LH-β[3] Follistatin**
Hausman et al., 2008	Outer and middle subcutaneous	Pig	90, 150, and 210 days	cDNA microarray from 560 pig gene sequences and RT-PCR**	Follistatin*
Yang et al., 2003	Abdominal omental	Human	59 years	cDNA microarray from EST[1] libraries and RT-PCR*	FSH-β[2] LH-β[3]
Flanagan et al., 2009	Subcutaneous and omental	Human	Middle aged (average 40)	qPCR and Western Blot	Follistatin
Zinger et al., 2003	Breast	Human	Middle aged (average 40)	RT-PCR, PRL bioassay	Prolactin
Hugo et al., 2008	Subcutaneous and omental	Human	Middle aged (average 40s)	Bioassay for PRL secreted in cell culture media	Prolactin

[1]Expressed sequence tags [2]Follicle-stimulating hormone β-subunit, [3]Luteinizing hormone β-subunit, [4]Visceral adipose tissue, [6]Glycoprotein hormone alpha chain

* Follistatin transcript validated by real time reverse transcriptase-polymerase chain reaction in middle subcutaneous adipose

** Identified in either proteomic or conventional protein assays

(EST) libraries (Table 1). The reproductive hormone transcripts expressed in the adipose included FSH-β and LH-β. The presence of these transcripts in human adipose tissue supports the possibility that adipose may have a reproductive endocrine role as obese humans suffer infertility.

An exhaustive review of the literature revealed a few protein studies where hormones important for reproduction or lactation were reported to be present in or secreted by adipose tissue. In 2009, Flanagan et al. studied the role of follistatin, a hormone that suppresses FSH, in human white adipose tissue and obesity (Table 1). They collected subcutaneous and omental adipose tissue from obese and non-obese women undergoing surgery and studied expression levels by quantitative PCR. White subcutaneous adipose tissue was reported to contain mRNA for follistatin and expression levels decreased with obesity. Differences were also reported between adipose depots, with subcutaneous adipose having higher follistatin levels than visceral. In addition to transcript presence, the follistatin protein was also present in adipose samples by evidence of western blotting. Additional efforts determined that the secretion of follistatin in vitro increased over time. Zinger et al. (2003) demonstrated that human breast adipose tissue expresses the transcripts as well as secretes prolactin at an increasing rate over time (Table 1). Given that the patients were likely not lactating based on their age, these findings indicate that the prolactin present in their breast adipose tissue may be carrying out one of its numerous other roles. Hugo et al. (2008) demonstrated prolactin release from human adipocytes and tissue explants derived from omental and subcutaneous depots in obese and non-obese men and women (Table 1). They reported a time-dependent release in vitro consistent with the

findings of Zinger et al. (2003). Hugo et al. observed a significant inverse relationship between total protein release and BMI for subcutaneous, but not visceral, adipose explants in all subjects pooled together. However, prolactin production by adipose tissue is many times less than production levels seen in pituitary cells. Whether prolactin secretion would have a significant impact systemically remains unclear however, Hugo et al. speculated that low prolactin levels may be sufficient to affect local tissue adjacent to adipose tissue.

Findings of reproductive hormone transcripts and proteins in adipose tissue provide the foundation for the possible reproductive role adipose may play. These results are suggestive that adipose may act as a reproductive gland; however a more detailed investigation is needed to determine if this is possible, particularly in cattle which have not been studied. This species provides agricultural products which rely heavily, if not solely, on reproduction. Additionally, cows have been utilized as a model for human reproduction due to the similarities in ovarian structure, follicular growth and waves, and the number of waves per cycle (Adams and Pierson, 1995; reviewed by Baerwald et al., 2012). The possible link between adipose tissue producing reproductive hormones and infertility has been glanced over and requires further attention and research. Depending on the extent to which hormones are present in and/or secreted by adipose tissue, this could lead to over secretion in the obese state.

Possible Consequences of Adipose Production of Reproductive Hormones

Adipose secreting reproductive hormones could explain why obesity has such a negative impact on reproduction in females. If adipose located around the reproductive tract is secreting these reproductive hormones, they could be acting on local receptors

on the ovary, oviduct, or uterus. Increased levels of follicle-stimulating hormone (FSH), the gonadotropin that supports growth and maturation of follicles (reviewed by Gloaguen et al., 2011), has been associated with reduced pregnancy rates and increase miscarriage in women undergoing in vitro fertilization (Scott et al., 1989). High levels of luteinizing hormone (LH), the gonadotropin critical for ovulation and formation of the corpus luteum (Luo et al., 2011), have been shown to alter normal oocyte development in women (Stanger and Yovich, 1985; Tarlatzis et al., 1995), reduce fertilization rates (Stanger and Yovich, 1985; Watson et al., 1993; Homburg, 1998), increase miscarriage (Regan et al., 1990; Homburg, 1998), and decrease pregnancy rates (Watson et al., 1993).

Along with the gonadotropins, prolactin and follistatin have been shown to have negative consequences on reproductive function when found in high levels. Prolactin is necessary around the time of parturition for mammary gland development and lactation (reviewed by Bachelot and Binart, 2007). In mice, it is also crucial for the maintenance of the corpus luteum and progesterone production (reviewed by Bachelot and Binart, 2007). Increased prolactin levels in women may inhibit the cyclic secretion patterns of FSH and LH, decrease the gonadotropin stimulation of the ovaries (Corenblum et al., 1976), and decrease progesterone production from the corpus luteum, causing a short luteal phase (Del Pozo et al., 1979). Follistatin acts to suppress FSH, and prevents the binding interaction of activin and myostatin (Flanagan et al., 2009). Since activin is important in follicular development, FSH secretion, and insulin secretion, too much follistatin has negative effects on reproductive function (Flanagan et al., 2009).

Follistatin, when in high concentrations, has been shown to arrest folliculogenesis in ovaries (Eldar-Geva et al., 2001).

Much of the infertility problems seen when these hormones are in high concentrations parallel the infertility problems associated with obesity. Providing that these hormones are produced from adipose tissue, an overly fatty state could potentially increase hormone levels. If this does not occur systemically, it could likely occur locally.

Systemic Levels of Reproductive Hormones Observed in Obese State

Knowing the adverse reproductive effects of hormones in high concentrations is critical, but only when the systemic reproductive hormone levels in obese individuals are also taken into consideration. Circulating LH levels in women have been reported to decrease (Grenman et al., 1986; Paradisi et al., 1986; Kaye et al., 1991; Malacara et al., 2001; De Pergola et al., 2006) or be unaffected (Sathya et al., 2010) by obesity. Circulating LH levels in men have been reported to be unaffected by obesity (Glass et al., 1977; Amatruda et al., 1978; Schneider et al., 1979; Strain et al., 1982), except in the massively obese, where basal levels and mean peak amplitude was decreased (Giagulli et al., 1994). Additionally, plasma prolactin levels have been reported to increase in premenopausal obese women (Kok et al., 2004) and pre-pubertal obese girls (Genazzani et al., 1978), but were not different in obese versus lean male rats (Finkelstein et al., 1986). Follistatin levels have been reported to increase (Eldar-Geva et al., 2001; Chen et al., 2010) while LH levels have been reported to decrease (Silfen et al., 2003) in women with polycystic ovarian syndrome compared to controls. Disparity of results may be due to different methods of quantification or differing ages/reproductive statuses in the subjects tested.

If adipose tissue is producing and secreting reproductive hormones, systemic levels in obese individuals could be increased, however, changes in hormone levels may not be observed if adipose utilizes a local production and delivery system. Also, the levels of hormones adipose may be capable of producing might not be high enough to observe a marked systemic increase.

Adipose Associated with the Reproductive Tract

Adipose tissue is located in specific regions throughout the body including subcutaneous, located beneath the skin; peri-renal, surrounding the kidneys; and visceral, which surrounds the organs in the abdomen. While these adipose depots are commonly known and discussed, less is known about adipose tissue associated with the reproductive tract. The periovarian fat pad, the depot surrounding the ovaries, uterus, and bladder (Ludgero-Correia et al., 2012), has been used to assess visceral adiposity in female mice (Gilbert and Ryan, 2011). In the male mouse, adipose tissue associated closely with the epididymis was compromised of larger adipocytes compared to other depots in the body (Caesar et al., 2010). The control of gene expression differed between epididymal, subcutaneous, and mesenteric adipose depots (Caesar et al., 2010). Additionally, Caesar and coworkers (2010) discovered functional differences between the proximal and distal epididymal adipose tissue such as reduced fatty acid synthesis and accumulation in proximal when compared to distal epididymal and mesenteric adipose. These findings support the notion that not all adipose tissue within the body is the same.

While there is evidence that mice have distinct adipose depots in close proximity to reproductive organs, an exhaustive review of literature provided few papers

describing this sort of depot in any non-rodent species. In a 2003 paper by Jankowska et al. looking at the broad ligament of cows, they mentioned having to remove adipose tissue in order to visualize the vessels, but did not characterize this adipose. Adipose tissue in critical locations, such as near the oviducts and ovaries, could provide a route for endocrine or paracrine hormone delivery. In endocrine delivery, the hormones enter the bloodstream and are then delivered to their target tissue. In paracrine delivery, hormones are delivered directly to neighboring cells. Adipose-secreted products, such as possibly reproductive hormones, could be using either route to access reproductive tissues.

Possible Routes for Hormone Transport in Reproductive Tissues

The vasculature of the support structures for the uterus, ovary, and oviduct create a localized endocrine environment different from systemic (Kotwica et al., 1983; Stefańczyk-Krzymowska et al., 2002; Jankowska et al., 2003). There are vessels and lymphatic tissue in the mesosalpinx, mesovarium, and mesometrium, structures that support the oviduct, ovary, and uterus, respectively. Stefańczyk-Krzymowska et al. (2002) showed a higher concentration of progesterone in local vasculature of the uterus compared to the systemic blood concentrations in pigs. In 1983, Kotwica et al. found that ^{3}H-PGF$_{2\alpha}$ injected into the uterus was able to reach the ovary via the mesosalpinx. If the adipose tissue associated with the reproductive organs and/or supportive structures secretes reproductive hormones into this local endocrine transport system, this would provide a route by which those hormones could have effects without being detectable in the systemic circulation.

Summary

Obesity has been strongly correlated with reproductive problems in various species over the years. Some of these problems may be explained, in part, by adipose tissue's role as an endocrine organ. Along with many adipokines, adipose may also produce reproductive or lactation hormones such as LH, prolactin, and follistatin. Reproductive or lactation hormones from adipose tissue may have local effects, thus of particular interest is adipose in close proximity to reproductive and mammary tissues. In order to further investigate the potential role of adipose tissue in reproduction, the working hypothesis of this thesis is that bovine adipose tissue may contain transcripts for and may secrete reproductive or lactation hormones that could, in turn, be directly affecting reproductive or mammary functions.

To test this hypothesis, the overall objective was to determine the extent to which adipose tissue in the bovine produces reproductive or lactation hormones previously detected to be transcribed in adipose tissue. As a first step, the amount of adipose tissue associated with reproductive tracts from mature bovine females was examined. After establishing the presence of adipose tissue on reproductive tracts, the next step was to determine the presence of specific reproductive hormone transcripts in subcutaneous, visceral, peri-renal, and mesosalpinx adipose depots. Different adipose depots throughout the body were chosen to examine because different depots have been shown to function differently. For example, different mRNA expression levels of leptin, insulin receptor, PPAR-γ, GLUT4 (Lefebvre et al., 1998), and angiotensinogen (Dusserre et al., 2000) have been reported between subcutaneous and visceral depots. This transcript effort was first examined in adipose originating from a non-descript pool

of mature cows. After being convinced of their presence, reproductive hormone transcripts were examined in the four previously mentioned adipose depots in mature cows of varying adiposity, selecting for homogeneous breed and reproductive status. Two groups of body condition were chosen because adipocytes can function differently based on size. For example, very large adipocytes secrete higher levels of leptin, adiponectin, IL-6, IL-8, TNF- α, and other chemokines when compared to very small adipocytes within an individual (Skurk et al., 2007). To determine the homology of the cows utilized, histological studies of the adipose tissue found in the mesosalpinx depot were compared to that in the subcutaneous depot for adipocyte size. Blood samples were also evaluated to determine the reproductive and metabolic/nutritional status of the cows. For prolactin and luteinizing hormone, efforts were made to determine whether presence of transcripts was indicative of presence of protein.

CHAPTER 3

MATERIALS & METHODS

Presence of Adipose Tissue on Mature Female Bovine Reproductive Tracts

Association of Adipose Tissue with Reproductive Tract

To assess the association of adipose tissue with the reproductive tract, tracts from mature females were collected from a local abattoir in November, 2010 (Southeastern Provision, LLC, Bean Station, TN, USA, n = 22 tracts total). Cows processed at this facility generally include 40% Angus or Angus cross, 25% Holstein, 10% Longhorn, 7% Hereford, 6% Charolais, 5% Brahman, 5% Jersey, and 2% other (including Brown Swiss and Guernsey; personal communication, Mr. James Brantley, Bean Station, TN). Non-pregnant reproductive tracts were collected from a random sampling of cows. They were observed for presence of adipose tissue and its location was recorded. Reproductive tracts were assigned two scores based on the general amount of adipose tissue present in the mesometrium and mesosalpinx, the broad ligament supporting the uterus and oviduct, respectively (scores ranged from 1 to 5, where 1 = minimal or none, 2 = slight, 3 = moderate, 4 = very abundant, and 5 = copious).

Approximating Adipose Tissue Amounts Associated with Female Reproductive Tracts

Non-pregnant tracts (n = 35) were collected on four different days from a random sampling of cows in order to determine amount of adipose tissue associated with mesosalpinx and mesometrium (Southeastern Provision, LLC). Reproductive tracts were first scored for adiposity as previously described. Adipose tissue was then removed from the reproductive tracts, keeping separate according to origin and location

(i.e., left mesosalpinx, left mesometrium, right mesosalpinx, and right mesometrium), and was weighed (g). Mesometrium adipose was examined in 10 of the tracts, while mesosalpinx adipose was examined in all 35. Mesosalpinx adipose was located directly below the oviduct within the supportive mesosalpinx tissue.

Abundance of LHβ, CGA, PRL, FST, & LEP Transcripts in Adipose Tissue Obtained from a Non-Descript Pool of Cows

Others have reported the presence of reproductive or lactation hormone transcripts pig subcutaneous (reviewed by Barb et al., 2005; Hausman et al., 2006; Hausman et al., 2008) and human visceral (Yang et al., 2003b) and breast adipose tissue (Hugo et al., 2008). The intent herein was to determine the extent to which similar transcripts may be present in adipose associated with the reproductive tract, namely the mesosalpinx. Additionally, visceral, subcutaneous, and peri-renal adipose depots were also collected and examined. Transcripts of interest included LHβ, CGA, PRL, FST, and leptin (LEP; “positive” control).

Sample Collection

Adipose tissue samples were collected between June to July 2011 from one Holstein, one Hereford, and seven Angus cows. Body condition scores were assigned to cows in the holding pen as per Richards et al. (1986) . Scores ranged from 1 to 9, where 1 = emaciated with prominent ribs and tail head, 2 = poor, 3 = thin with some fat over the spine, but no ribs identifiable, 4 = borderline, 5 = moderate with fat over the ribs and spine, 6 = high moderate, 7 = good, 8 = fat, and 9 = extremely fat with no visible bone structure. Cows utilized were not pregnant, with only seven of the nine cycling based on the presence of a corpus luteum. Adipose was collected from subcutaneous

(behind left shoulder at 6th rib), visceral (in the omentum covering the rumen), peri-renal (surrounding the kidney), and mesosalpinx (below the oviduct) depots. Small pieces (0.5 to 1 mg) of each adipose depot were snap frozen on site, and then stored at -80°C until total RNA isolation. Samples of ovary, kidney, and muscle tissues were also collected from random cows and processed in the same manner for comparison to adipose tissue. Pituitaries frozen immediately after collection were obtained at a different abattoir (Brown Packing Co., Inc., Gaffney, SC, USA).

RNA Isolation

Total RNA was extracted from frozen adipose and muscle samples using the RNeasy® Lipid Tissue Mini Kit (QIAGEN Inc. Valencia, CA, USA) as per the manufacturer's protocol, with one minor modification. Twice the amount of tissue and lysis reagent was run through each column (200 mg and 2 mL, respectively) in an effort to increase RNA yield. RNA was extracted from kidney, pituitary, and ovary using RNeasy® Maxi Kit (QIAGEN) as per the manufacturer's protocol. Concentration and integrity of RNA were evaluated by the NanoDrop® ND-1000 (ThermoFisher Scientific; Wilmington, DE, USA) and Agilent 2100 Bioanalyzer using a Nano Chip (Agilent; Santa Clara, CA, USA), respectively. Samples with degradation or insufficient concentration were not utilized.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Reverse transcription and quantitative PCR analysis was used to determine the relative abundance of transcripts within adipose tissue depots, as per Payton et al. (2011) with minor modifications. To ensure samples were DNA-free, RNA from each

sample was reverse transcribed (one reaction per cow) in the absence of enzyme then subjected to PCR with 18S primers. Reverse transcription (RT) was performed with 500 ng oligo d(T) primers (Fermentas; Glen Burnie, MD, USA), 400 units MMLV reverse transcriptase (Promega; Madison, WI, USA), 500 nM dNTP mix (ThermoFisher Scientific), and 50 units SUPERase-in (Life Technologies; Carlsbad, CA, USA) in 1X MMLV buffer to a final volume of 50 µL.

Primer sets were designed using Fast PCR (Version 6.1; Institute of Biotechnology; University of Helsinki, Finland) from bovine sequences in GenBank (Table 2). When feasible, primer sets were designed to span an intron. Primer concentration and annealing temperature were adjusted so efficiency was between 90 and 110% (where efficiency = $10^{(-1/slope)} – 1$; slope of –log concentration of standard curve versus threshold cycle; Table 2). PCR products were sequenced to ensure specificity of primers. Relative quantification was performed using Power SYBR Green PCR Master Mix (Life Technologies) in a 25 µL volume. Reverse transcribed total RNA (50 ng) from each sample was analyzed in duplicate in a 7300 ABI Real-Time PCR System (Applied Biosystems) for 45 cycles of denaturation for 15 sec (94°C), annealing for 30 sec (see Table 2), and extension for 30 sec (72°C), followed by a dissociation curve. Transcripts were considered to be present when crossing threshold cycle (C_t) at or before 36, as one copy of a transcript in a sample will cross threshold at cycle 36 (Bustin, 2004). Average C_t values were analyzed to determine whether transcripts were present in the samples.

Table 2: Sequences of primers used for qPCR amplification of genes of interest.

Gene	GenBank accession number	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size (bp)	Final primer concentration (nM)	Annealing Temperature (°C)
18S rRNA	AY779625	aagacggaccagagcgaaag	ggtcggaactacgacggtatct	100	200	60
LHβ	NM_173930	acccaatggtctccttccccgt	gtggtcacaggccaagggtt	107	200	58
CGA	NM_173901	agaacatcacctcggaagct	cactcggtgtggttctccac	94	200	60
PRL	NM_173953	cccttcctaccccggaagat	ggtcattccaggagcgcagcaa	96	400	60
FST	NM_175801	aacctaccgcaacgaatgtg	cagaaaacatcccgacaggtc	108	200	61
LEP	NM_173928	aggatgacaccaaaaccctc	agtccaaaccagtgaccctc	102	400	58
GFP	---	caacttcaagacccgccaca	tctggtaaaaggacagggcca	102	750	58 to 61

Abbreviations: LHβ = luteinizing hormone beta subunit, CGA = glycoprotein hormone alpha chain, PRL = prolactin, FST = follistatin, LEP = leptin, GFP = green fluorescent protein

Examination of Transcript (LHβ, CGA, PRL, GH1, FST, & LEP) and Protein (LH, Prolactin, & GH) Abundance in Adipose Tissue from Mature Cows of Varying Adiposity

Sample Criteria and Collection

Adipose samples from subcutaneous, visceral, peri-renal, and mesosalpinx depots were collected from November 2011 to March 2012 at a local abattoir from mature Angus and Angus-cross cows as previously described (as per Figure 1). Blood samples were taken at exsanguination and placed immediately on ice. Teeth were examined as per Pace and Wakeman (1983) to provide a general estimate of age. Samples were from non-pregnant cows with a functional corpus luteum present on the ovary to indicate cyclicity. Body condition scores were assigned to cows in the holding pen as per Richards et al. (1986) . In total, adipose was collected from 10 cows; five with BCS 3 (each individual was given a score of 3) and five with BCS 6 (scores of each individual animal were 5, 6, 6, 6, and 7 with a mean BCS of 6).

Pictures were taken of each reproductive tract and ovaries were removed for staging. Small pieces of each adipose depot were snap frozen at -80°C on site. An additional sample from mesosalpinx and subcutaneous adipose depots was also placed into 10% Formalin for histological analysis. Frozen adipose samples were placed at -80°C until future use for RNA and protein analysis. Blood tubes were spun (2,000 rpm for 20 minutes at 4°C) and serum and plasma were frozen separately at -20°C until future use.

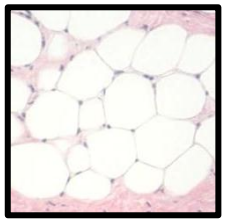
Collect Adipose Tissue from Mature Cows

BCS 6
N = 5

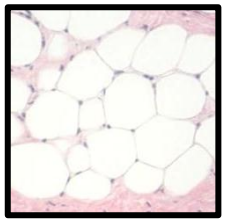
BCS 3
N = 5

Adipocyte Size

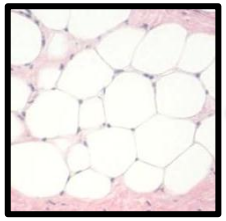
Adipose Tissue Depots



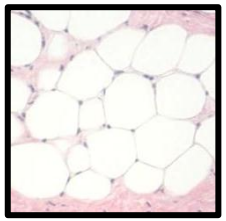
Subcutaneous



Visceral



Peri-renal



Mesosalpinx

Features of Total RNA
- Concentration
- rRNA ratio
- RIN

Transcript Presence
- Luteinizing Hormone - β
- Glycoprotein alpha chain
- Prolactin
- Growth Hormone
- Follistatin
- Leptin

Protein Presence
- Luteinizing Hormone
- Prolactin
- Growth Hormone

Blood Profiles
- Insulin
- Glucose
- NEFA
- β-Hydroxybutyrate
- Progesterone
- Prolactin
- Luteinizing Hormone

Figure 1: Schematic of experimental design and endpoints. Adipose tissue was collected from 10 mature Angus or Angus-cross cows in two body condition score (BCS) categories (BCS 3 or BCS 6). Adipose depots collected included subcutaneous, visceral, peri-renal, and mesosalpinx. Mesosalpinx and subcutaneous adipose was analyzed histologically for adipocyte size. RNA was extracted from all four depots and features of total RNA were analyzed. Total RNA was reverse transcribed and used to carry out quantitative PCR for 6 transcripts of interest. LH, prolactin, and growth hormone protein studies were performed on adipose tissue homogenates. Blood from each of the cows was analyzed for various parameters to determine nutritional/metabolic and reproductive status.

Histological Characterization and Adipocyte Size of Subcutaneous vs. Mesosalpinx Adipose Tissue from Mature Cows of Varying Adiposity

To determine adipocyte size, mesosalpinx and subcutaneous adipose tissues were fixed in 10% formalin. Slides were prepared and stained with hematoxylin and eosin by Jim Wesley (Ridge Microtome) for histological analysis, as previously described (Luna, 1968). Images were taken on a Nikon Eclipse E600 using the 10X objective. Adipocyte size was determined by the use of Image-J 1.46k (National Institutes of Health, USA). To correct for a halo effect from the light source, blank background images from each slide were added to each respective image. Images were converted to black and white (8-bit) and threshold was adjusted to maximize contrast between cell walls and background. The “close-” binary operation was applied to each image to smooth edges and fill in small holes. Adipocytes were counted for size (≈400 cells per depot) using the ROI manager and “wand” tool.

Examination of LHβ, CGA, PRL, GH1, FST, & LEP Transcript Abundance

RNA was extracted from subcutaneous, visceral, peri-renal, and mesosalpinx adipose samples as previously described. Features of total RNA (concentration, rRNA ratio 28S:18S, and RNA integrity number (RIN)) from subcutaneous, peri-renal, visceral, and mesosalpinx adipose were evaluated (Figure 1). Reverse transcription and qPCR methods also follow as previously described. Green fluorescent protein (GFP) RNA was spiked into RNA samples at a ratio of 50 pg per 2.5 µg of adipose RNA as an exogenous control used to normalize relative abundance. Growth hormone (GH1) mRNA was also examined using TaqMan® Gene Expression Assay (Applied Biosystems) as per manufacturer’s protocol (product size = 95 bp). Relative

quantification was performed using Universal TaqMan® PCR Master Mix (Applied Biosystems) in a 20 µL volume. Reverse transcribed total RNA (50 ng) from each sample was analyzed in duplicate in a 7300 ABI Real-Time PCR System (Applied Biosystems) for 45 cycles of denaturation for 15 sec (94°C), annealing for 30 sec (60°C), and extension for 30 sec (72°C). Bovine pituitary RNA served as the positive control for each gene except FST, which utilized bovine ovary RNA. To provide a comparison to tissues not normally associated with expression of the genes of interest, bovine muscle RNA was analyzed for PRL and CGA, while kidney RNA was analyzed for FST and LHβ. A pool representing 5 µL of RT from each adipose sample and control sample was created and serially diluted. This curve was run on each appropriate plate to determine the primer efficiency of each gene. Efficiencies were calculated from the curve and used to adjust C_t values before analysis. GFP was used to normalize each gene of interest and to ensure that the RT reaction was successful. To eliminate the inverse relationship between adjusted ΔC_t and abundance, adjusted ΔC_t values were subtracted from the maximum value possible. Values for abundance of transcripts ≤ 1 were at or near limit of detection except for PRL, where values ≤ 4 were at or near limit of detection due to less efficient primers.

Examination of Luteinizing Hormone, Prolactin, and Growth Hormone Protein

Protein was extracted from adipose tissue and pituitary samples as per Sánchez-Criado et al. (2006) with modifications. Samples were homogenized on ice in modified RIA buffer (0.5 M monobasic sodium phosphate buffer, 0.5 M dibasic sodium phosphate buffer, 0.135 M NaCl, 0.005 M NaHCO3, 0.01% Thimerosal;) containing Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA; 100 mg per

0.4 mL RIA for adipose and 100 mg per 5 mL RIA for pituitary) using a Power Gen 125 with 5x95 mm Generator (Fisher Scientific). Homogenates were spun for 10 minutes at 3,000g at 4°C and infranatant was collected. Protein concentration was determined by the FluoroProfile® Protein Quantification Kit (Sigma, Saint Louis, MI, USA) as per manufacturer's protocol using bovine serum albumin (BSA) as a standard (intra-assay coefficient of variance (CV) = 1.75%).

Luteinizing Hormone

Luteinizing hormone was measured in adipose tissue homogenates using a solid phase radioimmunoassay (RIA) as previously described by Moura and Erikson (1997) with minor modifications. Bovine anti-LH primary antibody was purchased from the NIDDK National Hormone and Peptide Program. Sensitivity of the LH assay was 0.03 ng/mL and average inter- and intra-assay coefficients of variation were 7.4 and 2.27%, respectively. Values were corrected for amount of protein loaded before analysis.

Prolactin

Adipose (125 µg) and pituitary (250 ng) homogenates were separated on 12% SDS-PAGE gels and electroblotted onto PVDF membrane. Western blot analyses were performed using the Snap ID system (Millipore, Billerica, MA, USA), as per manufacturer's protocol. Membranes were blocked using a 1:1 ChemiBLOCKER (Millipore, Billerica, MA, USA) and tris-buffered saline with 0.1% Tween 20 (TBST) mixture. Bovine anti-prolactin primary antibody (NIDDK National Hormone and Peptide Program) was used at 1:40,000 with the secondary antibody being a 1:40,000 goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate (Millipore; antibodies diluted in 1:1 ChemiBLOCKER/TBST). Western blots were developed using a chemiluminescence

substrate (Luminata Forte HRP Substrate; Millipore) following manufacturer's instructions for maximal quantity and time. Light-emitting bands were detected by exposing membranes to autoradiographic film for one hour.

Growth Hormone

Protein extraction, gel separation, and immunoblotting methods follow as stated above. Western blot analyses were performed using the Snap ID system (Millipore), as previously stated. Bovine anti-growth hormone primary antibody (NIDDK National Hormone and Peptide Program) was used at 1:40,000 with the secondary antibody being a 1:40,000 goat anti-human IgG HRP conjugate (Millipore; antibodies diluted in 1:1 ChemiBLOCKER/TBST). Western blots were developed as previously stated.

Blood Metabolites and Hormones

Blood samples were utilized to determine metabolic/nutritional and reproductive status of the 10 cows. (Figure 1) Serum non-esterified fatty acids (NEFA) levels were assessed by the Wako NEFA-HR(2) Microtiter Kit (Wako Diagnostics, Richmond, VA, USA) as per the manufacturer's protocol, with minor modifications. Sample and reagent amounts were halved due to plate well capacity. Average intra-assay CV was 1.10%. Serum beta-hydroxybutyrate (β-HB) levels were assessed by the BioVision β-hydroxybutyrate Assay Kit (BioVision, Mountain View, CA, USA) as per the manufacturer's protocol. Average intra-assay CV was 1.39%. Serum glucose levels were measured using the OneTouch® Ultra 2 glucose meter and Ultra test strips as per the manufacturer's protocol. Plasma insulin levels were assessed by a Bovine Insulin ELISA (Alpco Diagnostics, Salem, NH, USA) as per manufacturer's protocol with an intra-assay CV of 1.40%. Serum progesterone levels were assessed by the Coat-A-

Count® RIA (Siemens, Tarrytown, NY, USA) with an average intra-assay CV of 3.93%. Serum prolactin levels were assessed by a bovine-specific RIA as per Bernard et al., 1993 (average inter- and intra-assay CVs were 7.21 and 6.96%, respectively). Serum LH levels were assessed by RIA, as previously described.

Examination of Transcript (LHβ, CGA, PRL, FST, & LEP) and Protein (LH & Prolactin) Abundance in Mesosalpinx Adipose vs. Mesosalpinx Tissue

Examination of LHβ, CGA, PRL, FST, & LEP Transcript Abundance

As bovine mesosalpinx adipose tissue is sandwiched between two layers of broad ligament cells of the mesosalpinx, it is possible that mesosalpinx adipose samples collected for qPCR studies contained these other cells. In effort to clarify whether transcripts originated from broad ligament cells or adipose, additional samples were collected, separating cell tissue from adipose from five Angus or Angus-cross cows utilizing previous sample collection methods. Samples were collected in May, 2012 and all cows had body condition scores from 5 to 7. Methods for RNA isolation, reverse transcription, and qPCR were performed as previously described.

Examination of Luteinizing Hormone and Prolactin Protein

Protein was extracted from samples collected in summer 2012 as previously described. Luteinizing hormone was determined by RIA, as previously described. Samples were also evaluated for prolactin as previously described.

Statistical Analyses

Average C_t values for the non-descript pool of cows were analyzed as a completely randomized design (CRD) using mixed ANOVA model in SAS (SAS 9.2, SAS Institute, Cary, NC, USA) with fixed effects of depot. Features of bovine adipose

RNA were analyzed as a CRD using mixed ANOVA model in SAS with fixed effects of treatment, depot, and treatment by depot. Adipocyte size data were also analyzed as a CRD using mixed ANOVA model in SAS with fixed effects of treatment, depot, and the treatment by depot interaction. Quantitative PCR data were analyzed as a CRD using mixed ANOVA model in SAS with fixed effects of treatment, depot, and the treatment by depot interaction. Luteinizing hormone RIA data were analyzed as a CRD using mixed ANOVA model in SAS with fixed effects of treatment, depot, and the treatment by depot interaction. Blood variable data were analyzed as a CRD using mixed ANOVA model in SAS, with a fixed effect of treatment.

CHAPTER 4

RESULTS AND DISCUSSION

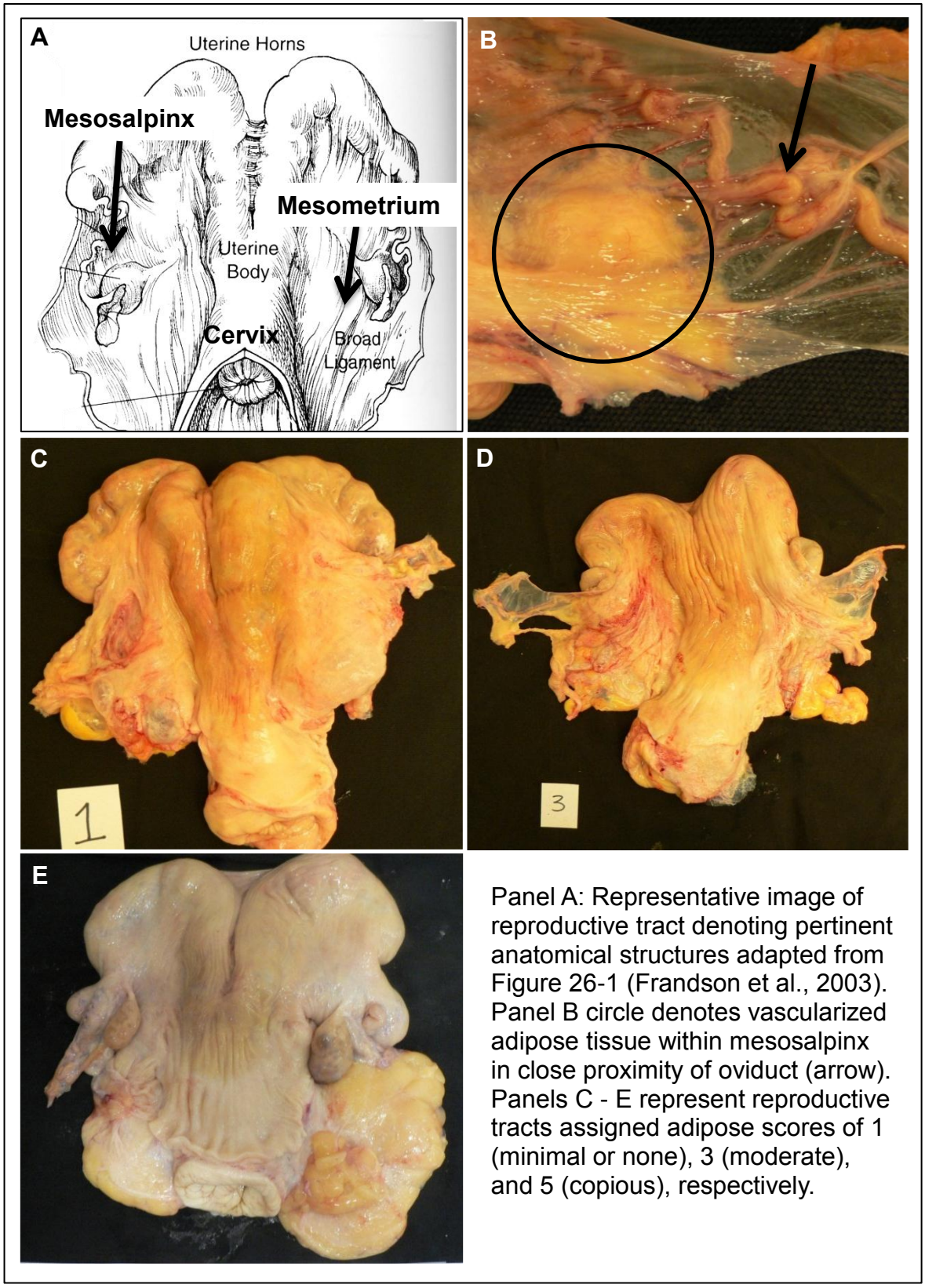
Presence of Adipose Tissue on Mature Female Bovine Reproductive Tracts

Association of Adipose Tissue with Reproductive Tract

Adipose tissue was observed on every reproductive tract obtained from a non-descript pool of mature females, with actual amounts ranging from minimal to copious. Figure 2 illustrates the scoring range and highlights the adipose seen associated with the mesosalpinx depot. Adipose tissue was observed in two consistent locations on reproductive tracts, though not every cow had adipose in both locations. The first location was the mesometrium, the tissue supporting the uterus (Figure 2A), though adipose tissue was generally not in close proximity to the uterus. Additionally, 14 out of 22 reproductive tracts examined had an adipose “pocket” observed in close proximity to the oviduct within the mesosalpinx tissue, the broad ligament supporting the oviduct (Figure 2B). When present, adipose was sandwiched between two layers of peritoneum and was vascularized (Figure 2B, circle). Reproductive tracts were assigned scores for mesosalpinx and mesometrium adipose depots and both depots had similar adiposity scores among the cows examined (Table 3).

Approximating Adipose Tissue Amounts Associated with Female Reproductive Tracts

In 34 of the 35 cows examined, adipose was associated with the reproductive tract. Adipose tissue was associated with the mesosalpinx in 80% of the reproductive tracts examined. In addition, all reproductive tracts whose broad ligament had not been removed during collection had adipose associated with the mesometrium. Reproductive



Panel A: Representative image of reproductive tract denoting pertinent anatomical structures adapted from Figure 26-1 (Frandson et al., 2003). Panel B circle denotes vascularized adipose tissue within mesosalpinx in close proximity of oviduct (arrow). Panels C - E represent reproductive tracts assigned adipose scores of 1 (minimal or none), 3 (moderate), and 5 (copious), respectively.

Figure 2: Representative images of adipose tissue associated with mature female bovine reproductive tracts.

Table 3: Adipose tissue scores in mesosalpinx and mesometrium associated with mature female bovine reproductive tracts.

	Mesosalpinx	**Mesometrium**
Mean	2.1	2.1
SEM	0.29	0.27
Range	1 to 5	1 to 5
n	22	22

Adipose Scores: 1 – minimal or none, 2 – slight, 3 – moderate, 4 – very abundant, 5 – copious

tracts were assigned scores for mesosalpinx and mesometrium adipose depots (Table 4) and the average scores for mesosalpinx and mesometrium were 2.60 and 1.90, respectively. Of those tracts having associated adipose, weights of individual mesosalpinx adipose depots ranged from 0.01 to 8.88 grams with an average total weight per reproductive tract of 3.69 ± 0.96 grams (Table 5). Not every tract had adipose on both sides, thus the two sides were weighed separately. The average weights for the left and right mesosalpinx adipose depots were 2.21 ± 0.49 and 1.90 ± 0.53 grams, respectively. Adipose tissue weights from individual mesometrium depots ranged from 0.36 to 10.97 grams with an average total weight per reproductive tract of 6.81 ± 1.99 grams (Table 5). The average weights for the left and right mesometrium adipose depots were 3.86 ± 1.57 and 3.54 ± 0.67 grams, respectively. The average total adipose tissue weight, including both sides and depots, was 9.62 ± 1.57 grams.

Summary

In total, 57 reproductive tracts from mature females were examined for presence of adipose tissue. Seventy-four percent of these tracts had adipose associated with at least one mesosalpinx (42/57), 57.4% had adipose associated with both mesosalpinges (31/54, 3 tracts had one oviduct cut off), and 26% had no adipose associated with the mesosalpinx (15/57).

These data clearly show that adipose tissue associates with the reproductive tracts of mature bovine females. Adipose was associated with two distinct tissues, namely the mesometrium, that which supports the uterus, and the mesosalpinx, that which supports the oviduct. While periovarian adipose tissue, that which is associated with the ovaries, in rodents has been described and utilized for study

Table 4: Adipose tissue scores in mesosalpinx and mesometrium associated with mature female bovine reproductive tracts.

	Mesosalpinx	**Mesometrium**
Mean	2.60	1.90
SEM	0.20	0.23
Range	1 to 5	1 to 5
n	35	30

Adipose Scores: 1 – minimal or none, 2 – slight, 3 – moderate, 4 – very abundant, 5 – copious

Table 5: Adipose tissue weights associated with mesosalpinx and mesometrium depots on mature female bovine reproductive tracts.

	Left Mesosalpinx (grams)	Right Mesosalpinx (grams)	Left Mesometrium (grams)	Right Mesometrium (grams)	Total Mesosalpinx (grams)	Total Mesometrium (grams)	Total Adipose (grams)
Mean	2.21	1.90	3.86	3.54	3.69	6.81	9.62
SEM	0.49	0.53	1.57	0.67	0.96	1.99	1.57
Range	0.02 to 8.88	0.01 to 8.44	1.07 to 10.97	0.36 to 5.58	0.02 to 17.32	1.43 to 14.30	6.71 to 14.30
n	28	21	6	8	25	6	6

(Ludgero-Correia et al., 2012), an exhaustive search of literature revealed no previous description of adipose tissue associated with the reproductive tract in the bovine. Jankowska et al. (2003) examined the broad ligament of cows after removal of adipose tissue in order to visualize the vessels, but did not characterize the adipose. Our findings are also novel in that adipose tissue was located in the mesosalpinx with a high degree of vascularity. This depot lies directly beneath the oviduct, a structure vital to fertilization and early embryonic development (Hunter, 2012). Adipose associated with the mesometrium was not located as closely with reproductive structures as documented in the mesosalpinx adipose depot.

These findings demonstrate that adipose is associated with the bovine reproductive tract in appreciable quantities. Mesosalpinx adipose tissue amounts varied greatly, but this is not unexpected considering the variety of cattle coming through the abattoir. Though amounts varied, the location of mesosalpinx adipose tissue remained consistent. This observation suggests that mesosalpinx adipose tissue can be considered as its own depot, though the origin of the adipocytes in this depot remains unclear. They may arise from preadipocytes that are always present in mesosalpinx tissue and only differentiate in response to certain metabolic signals. Hausman and Poulos (2004) demonstrated the presence of preadipocytes in the semitendinosus muscles of 5 and 7 day old pigs, providing evidence that preadipocytes may reside in non-adipose tissues. Further studies need to determine if preadipocytes are present within mesosalpinx tissue containing no visible adipose and if so, what signals trigger differentiation. Vascularized adipose tissue in this location could potentially deliver reproductive or lactation hormones to the reproductive tract.

Abundance of LHβ, CGA, PRL, FST, & LEP Transcripts in Adipose Tissue Obtained from a Non-Descript Pool of Cows

An initial effort was made to examine the presence of reproductive or lactationally important hormone transcripts in bovine adipose associated with the mesosalpinx. Additional adipose depots were examined as adipose can function differently based on location (Lefebvre et al., 1998; Dusserre et al., 2000; Fisher et al., 2001). To this end, the presence of LHβ, CGA, PRL, FST, & LEP transcripts in subcutaneous, visceral, peri-renal, and mesosalpinx adipose tissue depots was examined.

Transcripts for LHβ, CGA, PRL, FST, & LEP were present in adipose originating from subcutaneous, visceral, peri-renal, and mesosalpinx depots (Table 6). Per each transcript, there were no differences in the relative abundance observed in the adipose depots.

Collectively, these results demonstrate that transcripts important to reproduction (LHβ and CGA) and lactation (PRL) are present in appreciable amounts in bovine adipose associated with the reproductive tract (i.e., mesosalpinx) and subcutaneous, visceral, and peri-renal depots. While transcript presence is not always indicative of functional protein production, efforts demonstrated the presence of LEP, FST, and PRL transcripts, which are translated into protein by adipose tissue. Leptin protein is produced by subcutaneous and omental adipose tissue in humans (Russell et al., 1998; Van Harmelen et al., 1998; Machinal-Quélin et al., 2002) and subcutaneous, peri-renal, parametrial, and epididymal adipose tissue in rats (Bradley and Cheatham, 1999;

Table 6: Average C_t values in different tissue types of a non-descript pool of cows.

Adipose Origin	n	LHβ	CGA	PRL	FST	LEP
Subcutaneous	4	32.8	32.7	28.9	24.7	28.8
Visceral	2	30.3	31.2	26.0	23.5	26.2
Peri-renal	6	32.1	33.6	29.2	24.8	27.5
Mesosalpinx	5 (4 for CGA)	30.0	33.8	28.8	25.1	28.8
SEM		1.45	1.48	1.69	0.63	1.41
P-value		0.426	0.700	0.656	0.499	0.627
Tissue Origin						
*Kidney	1	32.1	35.2	36.7	29.5	40.3
*Muscle	1	28.3	39.7	39.5	27.6	.
*Pituitary	1	16.2	17.1	11.6	.	38.4
*Ovary	1	.	.	.	21.6	.

Abbreviations: LHβ = luteinizing hormone beta subunit, CGA = glycoprotein hormone alpha chain, PRL = prolactin, FST = follistatin, LEP = leptin

Tissues in which a transcript was not analyzed denoted by “.”

* Potential +/- “control” tissues analyzed for comparison to adipose tissue

Machinal et al., 1999). Follistatin protein is produced by human subcutaneous and omental adipose (Flanagan et al., 2009) as well as pig subcutaneous adipose (Hausman et al., 2006; Hausman et al., 2008). Prolactin protein is produced by human breast adipose (Zinger et al., 2003) and human subcutaneous and omental adipose (Hugo et al., 2008). Because LEP, FST, and PRL transcripts were also observed in the bovine adipose tissue examined, this attests to the possibility for bovine adipose to be serving as reproductive or lactationally important endocrine gland.

These findings are not likely unique to the four depots examined or to the bovine. The LHβ transcript has been observed in pig subcutaneous (Hausman et al., 2006) and human omental adipose (Yang et al., 2003b). The CGA transcript has also been observed in pig adipose (reviewed by Barb et al., 2005), with FST present in pig subcutaneous (Hausman et al., 2008) and human subcutaneous and omental adipose (Flanagan et al., 2009). Additionally, the PRL transcript has been reported in human breast adipose tissue (Zinger et al., 2003). It is highly possible that these transcripts may be more ubiquitous in adipose tissue, even among different species.

Depending on the extent to which adipose produces functional protein, the vasculature associated with the mesosalpinx adipose may allow for localized delivery. Protein levels may not be sufficient to alter systemic levels, but may provide fine tune regulation of reproductive or lactationally important tissues. All mesosalpinx adipose examined was highly vascularized with blood vessels leading clearly to the oviduct (Figure 2B). The oviduct is supplied with blood by many branches of the uterine and ovarian arteries; however the contributions from each artery vary greatly among species and even among individuals (reviewed by Garcia-Pascual et al., 1996). On their way to

the oviduct, these blood vessels would be located in the mesosalpinx, as this tissue supports the oviduct. Due to the unique location of the mesosalpinx adipose depot, any secreted products by the adipose tissue would likely have access to this local delivery mechanism, which would bypass systemic circulation. If proteins are present and secreted by adipose tissue, their target tissues may be in close proximity. For example, LH receptors have been reported on bovine corpora lutea (Weems et al., 2010) and oviduct (Sun et al., 1997). With a possible delivery mechanism in place and target tissues located nearby, it is plausible that adipose tissue is playing an important reproductive or lactation endocrine role.

Examination of Transcript (LHβ, CGA, PRL, GH1, FST, & LEP) and Protein (LH, Prolactin, & GH) Abundance in Adipose Tissue from Mature Cows of Varying Adiposity

Histological Characterization and Adipocyte Size of Subcutaneous vs. Mesosalpinx Adipose Tissue from Mature Cows of Varying Adiposity

Adipocyte size increases in relation to fatness (Hirsch and Batchelor, 1976; Spalding et al., 2008) and therefore, provides a useful measurement of degree of adiposity. In this study, cows were assigned a body condition score and then divided into two groups of differing adiposity (BCS 3 and BCS 6). There was no significant adiposity by depot interaction for adipocyte size (Table 7). Cows having a BCS 6 had larger adipocytes than cows with BCS 3 ($P < 0.0001$; Table 7, Figure 3). Adipocyte size did not differ between the mesosalpinx and subcutaneous depots (Table 7). Others have also shown no difference in adipocyte size between subcutaneous and peri-renal adipose depots in steers (Yang et al., 2003a). Interestingly, adipose images from cows

Table 7: Adipocyte size in subcutaneous and mesosalpinx adipose tissue of cows of varying adiposity.

	n	Adipocyte Size (pixels)
Adiposity		
BCS 6	5	10,605.30[a]
BCS 3	5	5,048.81[b]
SEM		535.73
P-value		< 0.0001
Depot		
Subcutaneous	10	7,687.08
Mesosalpinx	10	7,967.03
SEM		535.73
P-value		0.717
Adiposity x Depot		
BCS 6 Subcutaneous	5	11,034.22
BCS 6 Mesosalpinx	5	10,176.38
BCS 3 Subcutaneous	5	4,339.93
BCS 3 Mesosalpinx	5	5,757.68
SEM		757.64
P-value		0.153

[a,b]within a column differ

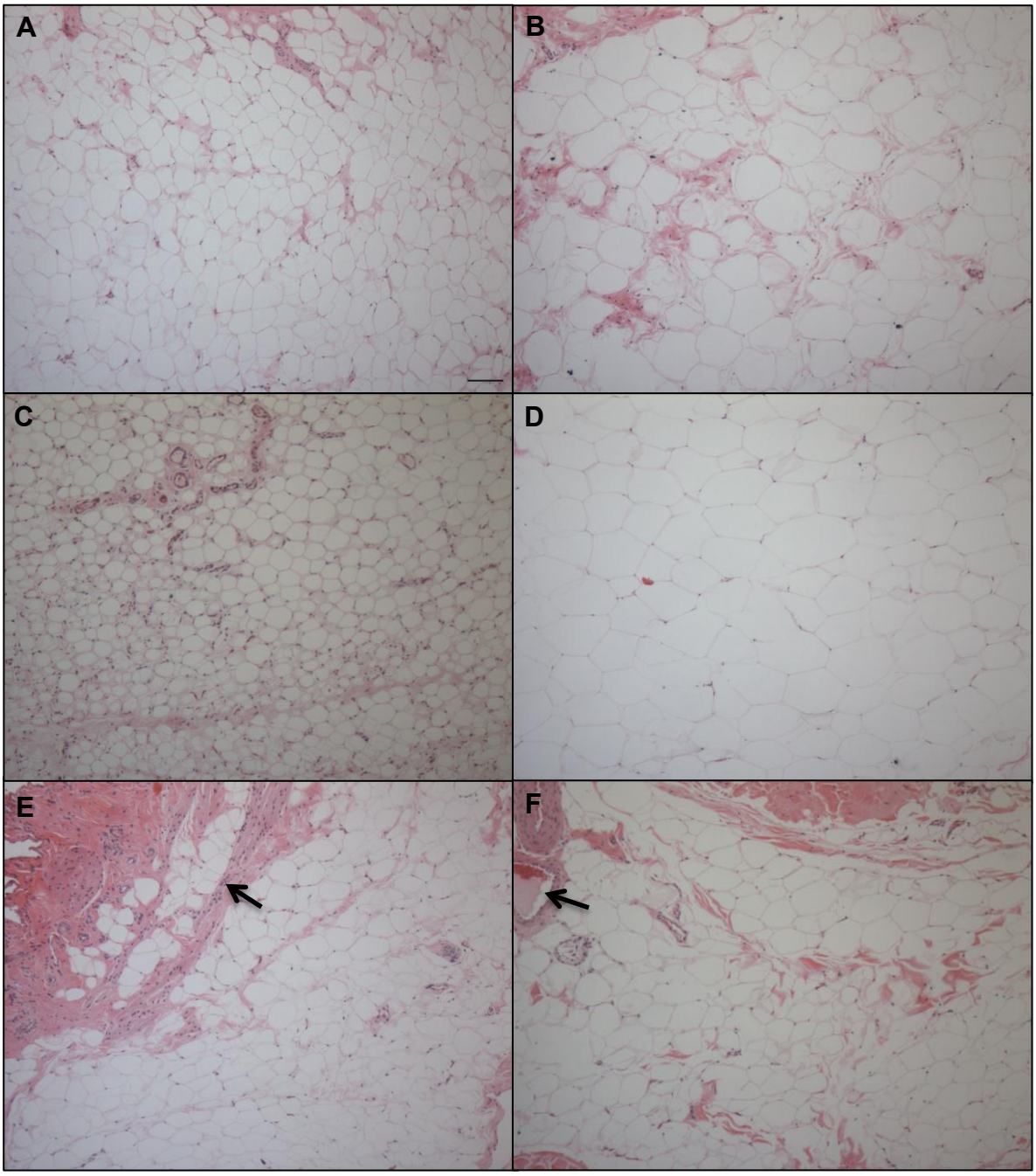

A
B
C
D
E
F

Figure 3: Representative images of mesosalpinx and subcutaneous adipose. The mesosalpinx or subcutaneous adipose from cows having BCS 3 versus BCS 6 (A & B, C & D, respectively). Arrow in Panel E points to what are likely broad ligament-derived cells in mesosalpinx adipose tissue. Arrow in Panel F points to small blood vessel in subcutaneous adipose tissue. Bar (Panel A) = 100 µm**.**

with higher BCS tended to be harder to analyze because incomplete cell walls prevented the software from counting many of the adipocytes (Figure 3B). This phenomenon may be biological as the triglyceride:protein ratio increases with adipocyte size (Radeau et al., 1998) or may be technical (e.g., due to incomplete penetration of the fixative into the tissue).

Histological images were also examined for other cell types. The majority of the adipose samples observed contained mostly adipocytes, characterized by large cells with lightly stained cell membranes and a single nucleus per cell (Figure 3A - D). Most mesosalpinx samples were either surrounded by or comingled with another cell type (Figure 3E, arrow) which were small in comparison to adipocytes and stained dark red. These other cells are likely the thin layer of broad ligament which surrounds the mesosalpinx adipose pocket. There were also instances of subcutaneous adipose containing cells other than adipocytes and both depots occasionally contained what appeared to be small blood vessels (Figure 3F, arrow). This latter finding was not unexpected due to the highly vascular nature of adipose tissue.

Features of Total RNA from Adipose Obtained from Mature Cows of Varying Adiposity

There were no adiposity by depot interactions for RNA extracted per mg tissue (P = 0.391; Table 8). However, less total RNA (ng/mg tissue) was recovered from adipose originating from cows having BCS 6 than those having BCS 3 (Table 8). The difference in RNA recovered from adipose in cows of varying adiposity agrees with Koranyi et al. (1990) who reported the same difference in epididymal adipose of obese and lean mice. In our study, different amounts of RNA were extracted depending on the depot (P = 0.001) with mesosalpinx adipose having higher abundance than all other adipose

Table 8: Features of total RNA from bovine adipose tissue from mature cows of varying adiposity.

	n	ng RNA per mg Tissue	rRNA	RIN
Adiposity				
BCS 6	19	1.37[b]	1.1	7.4[a]
BCS 3	20	2.70[a]	1.1	6.9[b]
SEM		0.26	0.05	0.15
P-value		0.001	0.628	0.018
Depot				
Subcutaneous	10	1.47[b]	1.2	7.4[a]
Visceral	10	2.02[b]	1.0	7.0[ab]
Peri-renal	9	1.05[b]	1.2	7.6[a]
Mesosalpinx	10	3.61[a]	1.0	6.7[b]
SEM		0.37	0.07	0.22
P-value		0.0002	0.175	0.023
Adiposity x Depot				
BCS 6 Subcutaneous	5	0.84	1.2	7.6
BCS 6 Visceral	5	0.82	1.1	7.2
BCS 6 Peri-renal	4	0.68	1.2	8.2
BCS 6 Mesosalpinx	5	3.15	0.9	6.8
BCS 3 Subcutaneous	5	2.10	1.2	7.2
BCS 3 Visceral	5	3.22	1.0	6.8
BCS 3 Peri-renal	5	1.43	1.1	7.0
BCS 3 Mesosalpinx	5	4.06	1.0	6.6
SEM		0.52	0.1	0.30
P-value		0.391	0.664	0.434

[a,b]within a column differ

depots. While difficult to explain, mesosalpinx adipose is encapsulated within two layers of cells comprising the broad ligament. Subcutaneous, visceral, and peri-renal adipose depots all had equivalent RNA concentrations extracted per mg tissue (Table 8) and did not appear to have any contaminating cells like the mesosalpinx depot.

Ribosomal RNA ratio (rRNA; 28S/18S) was examined in all samples. No differences were observed related to adiposity or depot (Table 8). Bahar et al. (2007) reported an rRNA ratio in bovine subcutaneous adipose tissue of 1.3 when collected at time of sacrifice, which is consistent with rRNA ratios repeated herein.

RNA integrity number (RIN), an RNA quality score based on the size distribution of RNAs present within a sample, was also examined. There were no adiposity by depot interactions observed (P = 0.434). The RIN values of RNA from adipose tissue of cows with BCS 6 were higher than those from cows having a BCS 3 (Table 8), suggesting that the size distribution of RNA may differ between adipose from cows of varying adiposity. Differences in RIN of RNA from different depots were also noted (Table 8), with subcutaneous and peri-renal adipose having higher values than mesosalpinx adipose. Whether adipose differences in RINs are biologically relevant or are a procedural artifact is unclear. Different tissues have been reported to have varying RIN values depending on the amount of connective tissue (Fleige and Pfaffl, 2006). It is important to note that efforts were taken to minimize the chance of artifacts in RNA samples. All samples were obtained and processed in a similar manner and stored at -80 °C until RNA isolation. Isolation of RNA utilized the exact technique for every sample and all adipose depots from one cow were isolated at the same time to decrease any procedurally related influences.

Examination of Transcript (LHβ, CGA, PRL, GH1, FST, & LEP) and Protein (LH, Prolactin, & GH) Abundance in Adipose Tissue from Mature Cows of Varying Adiposity

Differences in gene expression between small and large adipocytes have been noted (Jernås et al., 2006; Skurk et al., 2007), thus additional efforts were put forth to examine the influence of adiposity and different depots on the abundance of transcripts (LHβ, CGA, PRL, GH1, FST, & LEP) and protein (LH, Prolactin, & GH). There were no adiposity by depot interactions observed for LHβ, PRL, FST, or LEP (Table 9).

LHβ and CGA (Luteinizing Hormone)

Relative abundance of LHβ was similar in adipose originating from cows having BCS 3 versus 6, but differed depending on the adipose depot (Table 9). Higher levels of LHβ were noted in mesosalpinx adipose compared to other depots examined ($P < 0.0001$). There was a significant interaction between adiposity and depot for the relative abundance of the CGA transcript ($P = 0.057$). In BCS 6 cows, mesosalpinx adipose had less CGA than subcutaneous (Table 9). In BCS 3 cows, CGA in peri-renal adipose was marginally present. BCS 3 cows had lower CGA abundance in subcutaneous and peri-renal adipose than BCS 6 cows, while abundance in other depots did not differ between adiposity groups. Although CGA subunit possesses no biological activity, when combined with a molecule of LHβ this allows for the formation of luteinizing hormone (reviewed by Baenziger and Green, 1988). Differences observed in LHβ abundance between depots and CGA abundance between depots between adiposity groups may result in varying protein expression levels in these tissues and adiposity groups.

Table 9: Relative abundance of transcripts in adipose depots of mature cows of varying adiposity.

Effect		LHβ	CGA	PRL	FST	LEP
Adiposity						
BCS 6		4.9	2.7[b]	4.9	9.1[a]	11.0[b]
BCS 3		5.2	1.4[a]	5.0	9.6[b]	8.7[a]
	SEM	0.14	0.32	0.27	0.18	0.40
	P-value	0.143	0.007	0.686	0.056	0.0003
Depot						
Subcutaneous		4.3[a]	2.9	5.4[b]	10.3[b]	11.0[b]
Visceral		4.6[a]	2.2	5.6[b]	8.6[a]	10.2[b]
Peri-renal		4.4[a]	1.5	5.4[b]	9.7[b]	10.2[b]
Mesosalpinx		6.7[b]	1.5	3.4[a]	8.7[a]	8.2[a]
	SEM	0.20	0.45	0.38	0.25	0.57
	P-value	<0.0001	0.127	0.0005	<0.0001	0.008
Adiposity x Depot						
BCS 6 Subcutaneous		4.1	3.9[c]	5.2	9.9	12.7
BCS 6 Visceral		4.2	2.3[bc]	5.5	8.4	11.4
BCS 6 Peri-renal		4.7	3.1[bc]	5.4	9.3	11.4
BCS 6 Mesosalpinx		6.6	1.5[ab]	3.4	8.7	8.6
BCS 3 Subcutaneous		4.6	1.8[b]	5.6	10.6	9.3
BCS 3 Visceral		5.1	2.1[b]	5.7	8.8	8.9
BCS 3 Peri-renal		4.2	0.0[a]	5.5	10.0	8.9
BCS 3 Mesosalpinx		6.9	1.6[ab]	3.4	8.8	7.7
	SEM	0.28	0.63	0.54	0.35	0.80
	P-value	0.119	0.057	0.983	0.775	0.453

Abbreviations: LHβ = luteinizing hormone beta subunit, CGA = glycoprotein hormone alpha chain, PRL = prolactin, FST = follistatin, LEP = leptin

[a,b]within a column differ

Ct values were adjusted by the efficiency of the plate to account for differences in primer efficiencies, normalized with green fluorescent protein (GFP), and subtracted from the maximum value such that a larger number represents a higher abundance. Values for abundance of transcripts ≤ 1 are at or near limit of detection except PRL, where values ≤ 4 are at or near limit of detection due to less efficient primers.

To determine if the LHβ and CGA transcripts present in adipose tissue are translated and form the LH protein, an RIA was utilized. The majority of the samples for each adipose depot contained LH in detectable amounts (Table 10). Of those samples containing LH, there were no differences in the amount of LH per μg protein between BCS 3 and BCS 6 cows (P = 0.134) or between depots (P = 0.560). An exhaustive literature search revealed no reports of LH protein in adipose tissue, thus this may be the first evidence of such.

Whether LH is secreted from adipose tissue remains unclear, but if this is the case, LH could travel through the vasculature of the adipose to the vessels supplying the oviduct or ovary. As LH receptors have been reported on bovine corpora lutea (Weems et al., 2010) and oviduct (Sun et al., 1997), local target tissues could be affected by this delivery mechanism. Although LH is typically secreted by the pituitary and regulated by hypothalamic gonadotropin releasing hormone (GnRH), evidence suggests that non-hypothalamic GnRH as well as GnRH receptors are present in reproductive tissues (reviewed by Ramakrishnappa et al., 2005). If adipose tissue also contains GnRH receptors, it is plausible that local, non-hypothalamic production of GnRH may regulate LH secretion from adipose.

While mesosalpinx adipose contained a higher abundance of LHβ transcripts than all other adipose depots, this depot did not have higher levels of LH hormone. Like others have shown, this suggests that transcript abundance does not necessarily correlate with protein expression (reviewed by Maier et al., 2009). Future studies need to determine how much LH is produced from adipose tissue throughout the estrous cycle and whether these amounts are significant enough to alter systemic levels or

Table 10: Luteinizing hormone protein in adipose tissue of mature cows of varying adiposity.

	Frequency of Presence	pg per µg Protein	Range
Adiposity			
BCS 6	16/20	0.27	0.07 to 0.87
BCS 3	17/20	0.17	0.05 to 0.42
	SEM	0.04	
	P-value	0.134	
Depot			
Subcutaneous	9/10	0.28	0.05 to 0.87
Visceral	9/10	0.22	0.07 to 0.51
Peri-renal	10/10	0.23	0.06 to 0.53
Mesosalpinx	5/10	0.15	0.11 to 0.20
	SEM	0.06	
	P-value	0.560	
Adiposity x Depot			
BCS 6 Subcutaneous	4/5	0.44	0.28 to 0.87
BCS 6 Visceral	4/5	0.27	0.07 to 0.51
BCS 6 Peri-renal	5/5	0.22	0.10 to 0.53
BCS 6 Mesosalpinx	3/5	0.13	0.11 to 0.16
BCS 3 Subcutaneous	5/5	0.13	0.05 to 0.25
BCS 3 Visceral	5/5	0.16	0.07 to 0.33
BCS 3 Peri-renal	5/5	0.23	0.06 to 0.42
BCS 3 Mesosalpinx	2/5	0.16	0.11 to 0.20
	SEM	0.09	
	P-value	0.162	

produce a local effect. It is important to note that an effort was taken to ensure that all cows utilized were in diestrus (i.e., presence of a functional corpus luteum and progesterone levels above 1 ng/mL). It would, therefore, be difficult to say if the same results would be observed utilizing cows in different stages of the estrous cycle.

Prolactin

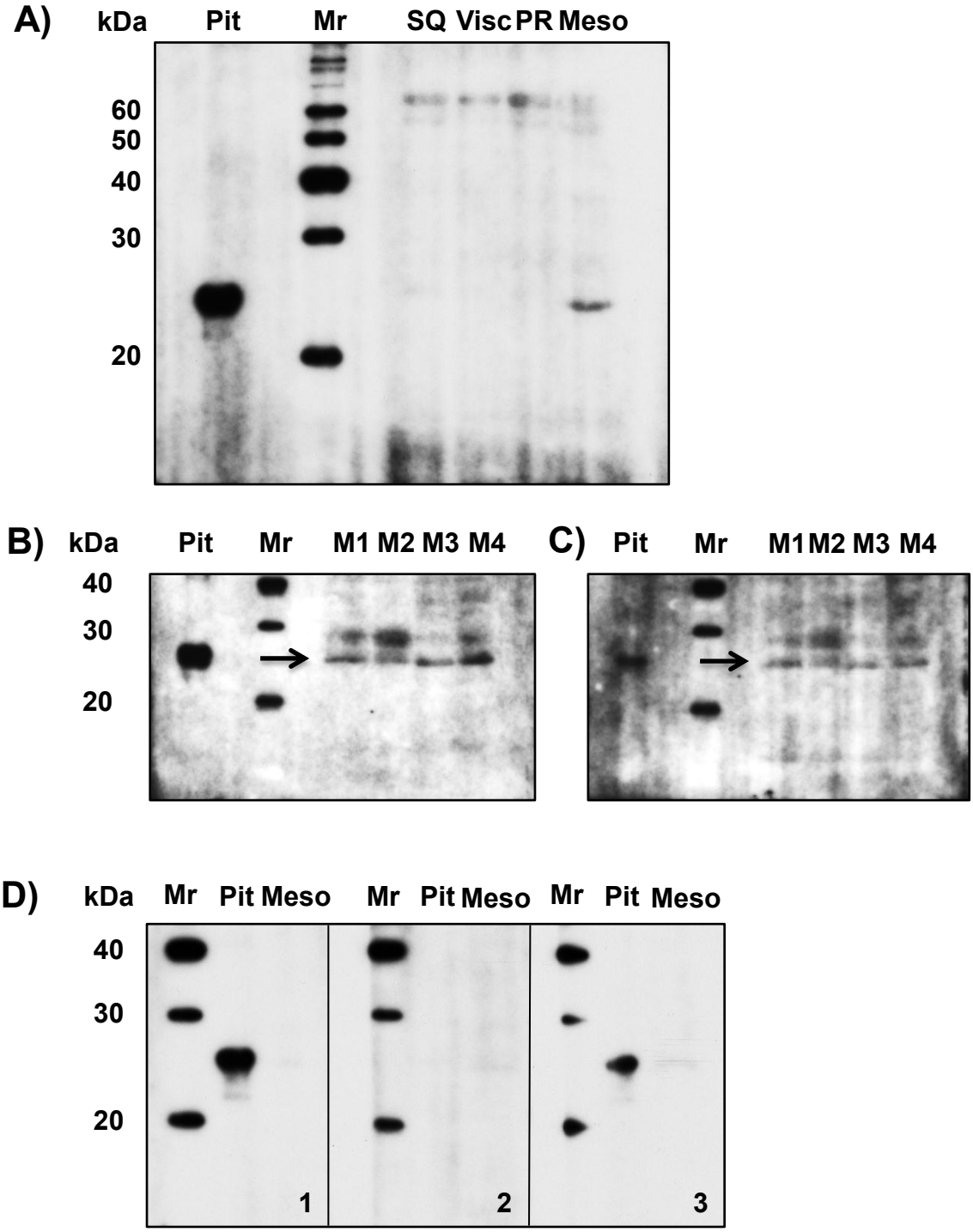
Abundance of prolactin transcripts did not differ between BCS 6 and BCS 3 cows (P = 0.686), however the mesosalpinx depot had lower abundance compared to other adipose tissues (P = 0.0005). Reduced transcript abundance in mesosalpinx adipose in this study is in contrast to results obtained using a non-descript pool of cows. Disparity between the two studies, while difficult to explain, may be related to differences in physiological states of the animals utilized. For instance, cows in this study were all in diestrus, whereas some of the cows in the first study were not cycling or may have been in a different stage of the cycle. Cows in early lactation are typically not cycling, so animals utilized in the first study could have been lactating. Additionally, samples from the non-descript pool were collected in June and July, while the cows of varying adiposity were collected between November and March. Prolactin levels in Holsteins are significantly higher in summer months compared to winter months (Koprowski and Tucker, 1973). Temperatures and planes of nutrition would likely be very different in these two seasons, which may have an effect on prolactin levels. Based on this evidence, additional efforts need to be made to determine if PRL abundance may be related to stage of lactation, season, or nutritional status.

After establishing the presence of prolactin transcripts in adipose tissue, prolactin protein presence was investigated using Western blotting to determine if transcript was

being translated. A band was detected at 22.1 ± 1.1 kDa in mesosalpinx adipose in 4/5 cows having BCS 3 and 2/4 cows having BCS 6 (Figure 4A). This band was similar in size to that observed in pituitary homogenate (22.2 ± 0.4 kDa). No band of similar size was detected in any of the other three adipose depots for the 10 cows examined. The pituitary band diminished greatly when primary antibody was immunoabsorbed with 3 µg/mL of a highly purified prolactin antigen, validating that this band was prolactin (Figure 4B & C). The mesosalpinx adipose bands also diminished slightly with this immunoabsorption effort (Figure 4B & C, arrow).

In addition to the 22.5 kDa bands observed, the antibody detected high molecular weight bands in many of the samples (78.9 ± 1.0 and 67.0 ± 2.0 kDa). A few adipose samples also contained a band at 27.9 ± 0.1 kDa. None of these bands were observed in pituitary and were extremely variable between cows and between depots. These large bands were suspected to be albumin based on the findings of Kelley et al. (1990), but immunoabsorbing with bovine serum albumin (BSA) did not consistently diminish the bands in question (data not shown). Due to low transcript abundance, it was expected that if prolactin protein was going to be present in adipose, it would be in low abundance. Techniques utilized to maximize the likelihood of detecting low abundance of prolactin may have introduced technical artifacts contributing to the immunoblotting inconsistencies observed. For the majority of the cows, a single distinct band was observed for mesosalpinx adipose consistent in size for prolactin.

It is interesting to note that while the PRL transcript was in marginal abundance in mesosalpinx adipose, this was the only tissue that contained a band of the correct size for prolactin protein. If the bands detected in mesosalpinx adipose are prolactin,


A)
kDa
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3

Figure 4: Analysis of prolactin protein in adipose by Western blotting. Representative immunoblot from single cow, after one hour exposure, with adipose depots and pituitary for positive control detected presence of appropriate sized band in mesosalpinx (Panel A). (Pit - pituitary, Mr - molecular weight marker, SQ - subcutaneous adipose, Visc - visceral adipose, PR - peri-renal adipose, Meso - mesosalpinx adipose). Mesosalpinx samples (M1 to 4) with pituitary were run on duplicate gels without (Panel B) and with (Panel C) preabsorption of primary prolactin antibody with 3 µg/mL highly purified prolactin antigen. Western blot analysis of pituitary and mesosalpinx adipose samples without (Panel D1) or with preabsorption with 10 µg/mL prolactin (Panel D2) versus preabsorption with 10 µg/mL growth hormone (Panel D3). Increased contrast by 20% on all images. Panels B and C also increased brightness by 20%.

the potential exists for a local effect on reproductive structures as prolactin receptors have been reported in bovine corpora lutea (Thompson et al., 2011), ovine follicles (Picazo et al., 2004), and ovine endometrial glandular epithelial cells (Stewart et al., 2000). While these reproductive tissues are in close proximity to mesosalpinx adipose in the bovine, caution must be taken in assigning possible roles of prolactin on these tissues without further studies. As prolactin has over 300 reported functions (reviewed by Bole-Feysot et al., 1998) and its receptor is expressed in nearly all tissues (reviewed by Goffin et al., 2002), it is difficult to say what effect it would have if translated and secreted by adipose tissue.

Growth Hormone

Because immunoabosrption efforts for prolactin were not sufficient to displace the entire band, efforts were taken to investigate another possibility for the identity of the protein detected. The prolactin antibody was made using highly purified pituitary-derived prolactin and cross-reacts with growth hormone at high concentrations, which is also ~ 22 kDa in size. Pituitary probed with prolactin antibody produced a specific single band (Figure 4D.1) that was completely displaced when the antibody was immunoabsorbed with prolactin antigen (Figure 4D.2). Immunoabsorbing prolactin antibody with growth hormone antigen diminished the pituitary band slightly (Figure 4D.3). This GH antigen was also highly purified from pituitary and may have been contaminated with prolactin. To evaluate the extent to which the band previously visualized with prolactin antibody was prolactin rather than growth hormone, we examined adipose for transcript and protein presence. Only 3 samples out of 39 had possible GH1 transcripts and values were at or below the limit of detection. Pituitary

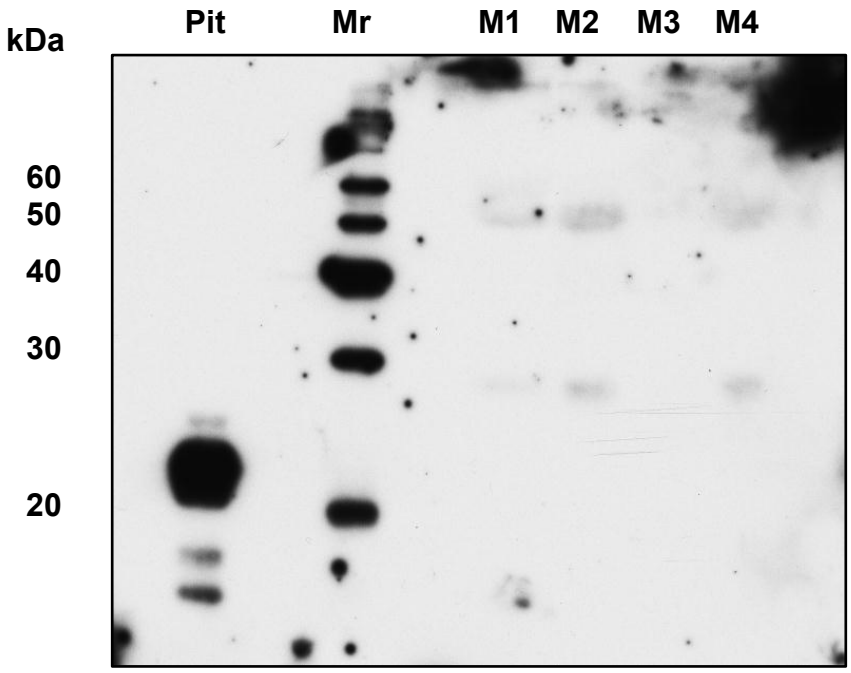
and 4 mesosalpinx adipose samples that had previously displayed a band at the correct size when probed with prolactin antibody were evaluated by Western blot for growth hormone presence. When probed with growth hormone antibody, a band at 21.7 kDa was detected in pituitary, which is the correct size, but corresponding bands were not detected in adipose samples (Figure 5A). Pituitary also contained bands at 15.7 and 13.6 kDa, while some adipose samples had bands at 54.9 and 29.6 kDa. When immunoabsorbed with highly purified pituitary-derived growth hormone antigen, the pituitary band was completely displaced (Figure 5B).

These results suggest that the bands being detected by prolactin antibody are not growth hormone. The non-specific bands could be due to the high protein amount and long exposure time as with the prolactin efforts. While immunoabsorption efforts reveal that the bands detected in mesosalpinx adipose samples by prolactin antibody may be prolactin, further efforts need to be made to confirm the identity.

FST

Cows with higher adiposity had lower abundance of FST transcripts ($P = 0.056$) and abundance also differed between depots (Table 9). Transcript abundance for FST was significantly lower in mesosalpinx and visceral adipose than in peri-renal and subcutaneous depots ($P < 0.0001$). The higher abundance observed in subcutaneous adipose when compared to visceral agrees with Flanagan et al. (2009) who reported the same results in humans. The higher abundance in cows having BCS 3 when compared to BCS 6 is also consistent with Flanagan et al. (2009) who reported higher FST expression in subcutaneous adipose of lean versus obese women. Because others have reported follistatin protein production and secretion by pig subcutaneous

A)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B)

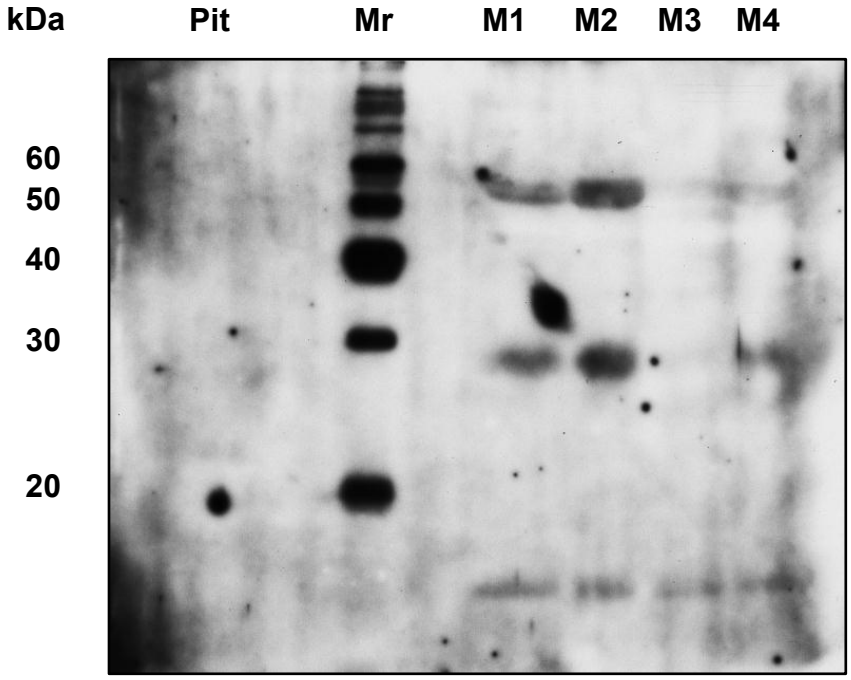
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Figure 5: Analysis of growth hormone protein in adipose by Western blotting. Representative immunoblot of mesosalpinx adipose samples run on duplicate gels without (Panel A) and with (Panel B) immunoabsorption of primary GH antibody with 10 µg/mL highly purified growth hormone antigen. (Pit - pituitary, Mr - molecular weight marker, M1 to 4 - mesosalpinx adipose from 4 different cows). Antibody detected presence of appropriate sized band in pituitary sample, but not in adipose samples. Increased contrast by 20% on all images.

(Hausman et al., 2006) and human subcutaneous and omental adipose (Flanagan et al., 2009), it is likely that bovine adipose examined may also produce and secrete follistatin. If follistatin is secreted by mesosalpinx adipose, it may inhibit FSH secretion, thereby suppressing follicular growth.

LEP

Abundance of LEP transcript was different between the two adiposity groups, with BCS 3 cows having lower abundance (P = 0.0003). These results agree with the literature as LEP abundance has been reported to be higher in large adipocytes when compared to small (Skurk et al., 2007). The cows having BCS 6, which had significantly larger adipocytes, had higher abundance of LEP mRNA than cows having BCS 3. Abundance also differed between adipose depots with mesosalpinx adipose having lower levels than all other depots (P = 0.008). One possible explanation for this difference may be that leptin from visceral, peri-renal, and subcutaneous adipose participates in metabolic status, while leptin from mesosalpinx adipose could potentially have a function focused on the reproductive tract. Others have reported production and secretion of leptin protein by subcutaneous and omental adipose tissue in humans (Russell et al., 1998; Van Harmelen et al., 1998; Machinal-Quélin et al., 2002) and subcutaneous, peri-renal, parametrial, and epididymal adipose tissue in rats (Bradley and Cheatham, 1999; Machinal et al., 1999), and this is also the case in the bovine (Kadokawa et al., 2007).

Blood Metabolites and Hormones

Various blood parameters were examined to assist in determining the nutritional, metabolic, and reproductive status of the animals utilized and to determine if adiposity

influenced the values (Table 11). Insulin, a hormone central to metabolic control, ranged from 0.1 to 0.35 ng/mL in plasma samples. These values are very consistent with the levels seen after a 30 hour fast in Angus bulls and steers (Ward et al., 1992) and since animals utilized were fasted at least to some degree due to travel, the observed insulin values agree. There were no differences in plasma insulin between BCS 6 and BCS 3 cows (P = 0.121). Another critical metabolic molecule and energy source, glucose, ranged from 82 to 392 mg/dL in serum and was not different between adiposity groups (P = 0.267). These levels were much higher than reported levels of 76 mg/dL (Schrick et al., 1990), especially considering cows were in a fasted state, most having not eaten for approximately 12 to 24 hours before slaughter. These unusually high levels could be due, in part, to the high amount of stress endured during the slaughter process, similar to increased glucose levels in horses during exsanguination seen in Werner and Gallo, 2008. Elevated glucose levels similar to what we observed have also been reported in steers transported by road for up to 15 hours (Warriss et al., 1995).

Serum non-esterified fatty acid (NEFA) levels are used to asses energy status in cattle, with elevated NEFA indicating negative energy balance (reviewed by Adewuyi et al., 2005). Serum NEFA levels ranged from 0.013 to 0.027 mmol/L and were not different between adiposity groups (P = 0.687). NEFA levels were low, especially for fasted animals. These results are perplexing, as Angus bulls and steers showed lower NEFA levels in the fed state (0.08 mmol/L) than their fasted counterparts (0.35 mmol/L; Ward et al., 1992). β-hydroxybutyrate, similar to NEFA, is a metabolite used to assess energy status in cattle with high levels indicating a negative energy balance

Table 11: Blood metabolites and hormones in mature cows of varying adiposity.

		Adiposity			
	n	**BCS 3**	**BCS 6**	**SEM**	**P-value**
Insulin (ng/mL)	10	0.14	0.21	0.03	0.121
Glucose (mg/dL)	9	123.00	195.80	42.58	0.267
NEFA (mmol/L)	9	0.02	0.02	0.002	0.687
β-HB (nmol/L)	9	12.76	11.85	3.04	0.839
Progesterone (ng/mL)	9	4.48	7.66	1.45	0.164
Prolactin (ng/mL)	9	139.28	107.50	53.67	0.689
LH (ng/mL)	8	1.17	1.64	0.16	0.081

Abbreviations: NEFA = non-esterified fatty acid, β-HB = beta-hydroxybutyrate, LH = luteinizing hormone

All parameters were determined in serum except insulin which utilized plasma

(reviewed by Ndlovu et al., 2007). Serum β-HB ranged from 6.49 to 22.64 nmol/L and levels were not different between adiposity groups (P = 0.839). In dairy cows, β-HB levels have been reported ranging from 0.27 to 3.90 mmol/L, with an average of 0.88 mmol/L (Nielen et al., 1994). Dairy cattle typically have high NEFA levels when they are in negative energy balance (Reviewed by Adewuyi et al., 2005), so our data indicate that the cows utilized in this study were not in negative energy balance. Beta-hydroxybutyrate levels were low, also indicating that the cows were not in negative energy balance.

Reproductive and lactation hormones were investigated to determine the physiological status of each animal. In order to confirm that each cow was cycling, progesterone levels were assessed. Serum progesterone ranged from 3.1 to 13 ng/mL and was not different between adiposity groups (P = 0.164). These levels are typical of values observed during the estrous cycle (Wettemann et al., 1972).

As a protein of interest in the adipose samples, prolactin was also measured in serum. Levels ranged from 12.3 to 308.3 ng/mL and were not different between adiposity groups (P = 0.689). These values, except the highest sample, are consistent with the literature, as serum prolactin levels have been reported ranging from 9 ng/mL in pregnant heifers up to 121 ng/mL in lactating dairy cows (Koprowski and Tucker, 1973). Prolactin levels varied greatly, likely due to varying stages of lactation for the group of cows.

Serum LH levels ranged from 0.9 to 1.9 ng/mL which is consistent with reported baseline levels of 0.7 – 1.9 ng/mL (Swanson and Hafs, 1971). Levels were not different between adiposity groups (P = 0.081), though a trend was present with BCS 6 cows

having slightly higher LH than BCS 3 cows. This finding suggests that if adipose secretes LH, it is likely not contributing to the systemic circulation. Luteinizing hormone in cattle stays at a low, pulsatile level and then spikes rapidly (8.1 ng/mL) at the time of estrus, prior to ovulation (Swanson and Hafs, 1971), indicating that the cows utilized in this study were not in estrus.

Examination of Transcript (LHβ, CGA, PRL, FST, LEP) and Protein (LH and Prolactin) Abundance in Mesosalpinx Adipose and Mesosalpinx Tissue from Mature Cows

Due to concerns that mRNA could be derived from contaminating broad ligament cells in mesosalpinx adipose tissue samples, an effort was made to examine the abundance of transcripts in both tissues. Effort was made to separate broad ligament tissue from the mesosalpinx adipose, such that adipocytes would only be present in the adipose samples. Each transcript was present in all of the mesosalpinx adipose and mesosalpinx broad ligament (hereafter referred to as mesosalpinx tissue) samples investigated, though mesosalpinx adipose only had marginal CGA and PRL (Table 12).

Mesosalpinx tissue had a higher abundance of LHβ than mesosalpinx adipose ($P = 0.051$), but there were no differences in CGA abundance between the two tissue types (Table 12). When mesosalpinx adipose and its surrounding mesosalpinx tissue were examined for LH protein using an RIA, 3/3 of each tissue type contained LH above the sensitivity of the assay (Table 13). No differences in LH per μg protein were detected between the mesosalpinx adipose and tissue ($P = 0.396$). The presence of LH in mesosalpinx adipose is surprising considering the marginal presence of CGA transcript in this tissue. The presence of LH in mesosalpinx tissue is also surprising,

Table 12: Relative abundance of transcripts in mesosalpinx adipose and mesosalpinx tissue of mature cows.

Tissue Type	LHβ	CGA	PRL	FST	LEP
Mesosalpinx Adipose	7.1[a]	1.0	3.9	9.6	10.3[b]
Mesosalpinx Tissue	10.1[b]	2.0	4.6	8.8	5.3[a]
SEM	0.95	0.40	0.59	0.29	0.8685
P-value	0.051	0.132	0.448	0.080	0.003

Abbreviations: LHβ = luteinizing hormone beta subunit, CGA = glycoprotein hormone alpha chain, PRL = prolactin, FST = follistatin, LEP = leptin

[a,b]within a column differ

Ct values were adjusted by the efficiency of the plate to account for differences in primer efficiencies, normalized with green fluorescent protein (GFP), and subtracted from the maximum value such that a larger number represents a higher abundance.

Values for abundance of transcripts ≤ 1 are at or near limit of detection except PRL, where values ≤ 4 are at or near limit of detection due to less efficient primers.

considering that this tissue is normally thought of as being a supportive structure for the oviduct. Whether the LH detected was from the broad ligament cells or was residual from any blood vessels within remains to be determined. That the adipose samples contained the same amount of LH per µg protein as the surrounding tissue provides evidence that LH is in in both mesosalpinx adipose and tissue.

There was no difference in abundance of PRL between mesosalpinx adipose and mesosalpinx tissue (Table 12), though both had marginal levels. When protein was examined using Western blotting, a band of 21.5 ± 0.6 kDa was detected in 4/4 mesosalpinx tissue samples and 3/4 mesosalpinx adipose samples. These bands were consistent with the pituitary bands, which were 22.1 ± 0.7 kDa in size. Immunoabsorbing with prolactin provided results similar to those previously observed (Figure 4). Additional higher molecular weight bands were also observed in this effort similar to previous efforts (69.3 ± 1.6 and 26.9 ± 0.07 kDa). Immunoabsorbing with BSA did not support our suspicion of these bands being albumin or degradation products of albumin.

There was no difference in abundance of FST between mesosalpinx adipose and mesosalpinx tissue (Table 12). The abundance of LEP was significantly higher in mesosalpinx adipose when compared to mesosalpinx tissue (Table 12), which is expected as leptin is highly abundant in adipose tissue. Presence of LEP transcripts in mesosalpinx tissue is novel, though the literature supports LEP expression in non-adipose tissue such as human placenta (Henson et al., 1998).

The presence of reproductive and lactation transcripts in the supportive mesosalpinx tissue is a novel finding, as an exhaustive search of the literature

Table 13: Luteinizing hormone protein in mesosalpinx adipose and mesosalpinx tissue of mature cows.

	Frequency of Presence	pg per µg Protein	Range
Mesosalpinx Adipose	3/3	0.15	0.11 to 0.20
Mesosalpinx Tissue	3/3	0.08	0.01 to 0.20
	SEM	0.05	
	P-value	0.396	

provided no publications documenting these results. These findings support our concern that the cells of the mesosalpinx could be contributing to the presence of the transcripts of interest in mesosalpinx adipose samples. The purpose of the broad ligament containing transcripts for reproductively or lactationally important hormones remains unclear, requiring further investigation.

SUMMARY/CONCLUSION/IMPLICATIONS

If adipose tissue plays an endocrine role in reproduction and/or lactation, many species could potentially be affected. Of particular interest are findings of reproductive and lactation hormones in adipose tissue of cattle, as this species requires successful and efficient reproduction and lactation in order to provide products for human consumers. Our efforts have characterized an adipose depot in the reproductive tract of the bovine in order to fill a gap in information. The close association of the mesosalpinx adipose tissue with the oviduct and the high degree of vascularity observed within this depot provide a possible route to deliver secreted hormones directly to target tissues.

The presence of reproductively and lactationally relevant hormone transcripts in four distinct adipose depots in the bovine provides evidence that adipose tissue has the framework necessary to produce these hormones. We reported luteinizing hormone protein in all four adipose depots and what seems to be prolactin protein in mesosalpinx adipose. Other transcripts observed in bovine adipose tissue have been reported to be translated and secreted by adipose tissue, thus this is likely the case for bovine adipose as well.

A crucial next effort needs to be culturing bovine adipose tissue in order to determine whether the hormones within are being secreted. Additionally, efforts should

be taken to determine if hormone levels secreted are high enough to affect systemic levels and to determine what regulates secretion. It would also be important to determine if vascular structures in mesosalpinx adipose connect to vasculature of the mesosalpinx. While results presented provide evidence for a reproductive or lactational endocrine role for adipose tissue, many more efforts are needed to determine the functional significance of such a role.

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