EXPRESSION OF PREGNANCY-SPECIFIC BETA-1-GLYCOPROTEIN, AROMATASE AND NITRIC OXIDE SYNTHASE IN BOVINE SPERM

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ABSTRACT

In the search for alternative indicators of fertility in bulls, this investigation evaluated the effects of breed, type of cattle and age, on the relative expression of the genes Aromatase, Pregnancy-Specific-Beta-1-Glycoprotein (PSG1), and Nitric Oxide Synthase (NOS) in bovine semen. Sperm from Jersey, Holstein, Brahman, Brangus, Charolais, Charbray, Senepol, and Simmental bulls was collected and extracted mRNA was analyzed for expression of the genes by using Reverse transcriptase and Real Time Polymerase Chain Reaction (RT-PCR). The relative expression of aromatase was lower ($P<0.07$) in sperm from Jersey bulls (0.89) than in bulls of all other breeds (average 1.01). The relative expression of PGS1 was greater ($P>0.02$) in sperm from dairy (1.00) than from beef bulls (0.96). Age did not affect relative expression of the genes investigated. Additionally, in data from thirty-one bulls, expression of NOS was positively correlated with scrotal circumference ($r=0.31$; $P<0.08$). To our knowledge, these are the first results showing that the genes aromatase, PSG1 and NOS can be detected in bull sperm and may have implications as molecular markers for bull fertility.

Key words: sperm, bovine, PSG1, NOS, Aromatase

RESUMEN

En la búsqueda de marcadores alternos para la fertilidad en los toros, se evaluó el efecto de la raza del toro, el tipo de ganado y la edad, sobre la expresión relativa de los genes Aromatasa, Glicoproteína Beta-1-Específica de la Preñez (GSP1), y Óxido Nítrico Sintasa (ONS) en espermatozoides bovinos. Se colectó semen de toros Jersey, Holstein, Brahman, Brangus, Charolais, Charbray, Senepol y Simmental para extraer el ARN mensajero y evaluar la expresión de los genes por la reacción de transcriptasa reversa y la reacción en cadena de la polimerasa en tiempo real. La expresión relativa de la Aromatasa fue menor ($P < 0.07$) en el semen de toros Jersey (0.89) en comparación a las otras razas (promedio 1.01). La expresión relativa de GSP1 fue mayor ($P < 0.02$) en el semen de toros lecheros (1.00) comparado a los de carne (0.96). La edad no tuvo efecto ($P > 0.05$) sobre la expresión relativa de los genes investigados. En adición, datos obtenidos de treinta y un toros revelaron que la expresión de ONS correlacionó directamente con la circunferencia escrotal ($r = 0.31$, $P < 0.08$). Hasta donde conocemos, estos resultados muestran por primera vez que la expresión de los genes Aromatasa, GSP1 y ONS puede ser detectada en semen bovino, lo que podría tener implicaciones para uso como marcadores moleculares para la fertilidad de los toros.

Palabras clave: semen, bovino, GSP1,ONS, Aromataza

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Chapter 1. INTRODUCTION

Animal production greatly contributes to the agricultural income of Puerto Rico; in 2011 it represented 47.81%. Of this percentage, 26.1% comes from the dairy industry and 3% from the beef industry. The dairy and beef industries had a gross income of $237,107,000, and $24,361,000 (División de Estadísticas, 2011), respectively. Recently, these two industries have suffered a decrease in production. For example, the dairy and beef industry in Puerto Rico suffered a revenue loss of 1% and 4% respectively (National Agriculture Statistical Service, 2011). Those percentages represent the loss of more than 30 million dollars for the country, and particularly, the farmers.

Some issues that are affecting the productivity of dairy and beef farmers are: high cost of fuel, feed, and utilities; and poor farm management. The latter is the only variable that the farmers has control over. Unfortunately, many farmers in Puerto Rico lack basic management practices such as good record keeping. Record keeping at both dairy and beef farms includes documenting levels of animal production, inventory, feed, and reproduction traits. Better organization of all of these records should help farmers had better management of the productivity and be cost effective.

In regard to reproduction, farmers need to account for the genetic selection of animals to establish traits that will eventually improve the productive performance of cattle. Today, with molecular biology techniques, cow fertility can be improved by indirect selection on the bull, by using molecular markers (Mackinnon et al., 1990). Moreover, molecular markers could be used as a tool to test for fertility traits and will give a higher economic value to the bulls (e.g. selling semen at higher prices). Bulls should be tested as soon as they reach puberty, to reduced costs of

maintaining undesired sires. Molecular markers could be part of the reproductive management practices in cattle.

A recent research discovered that sperm carries metabolites that contributes with important process like capacitation, fertilization and implantation (Rodriguez et al., 2005; Avendaño et al., 2009; Hamatani, 2012). For example, some researchers determined that the sperm carry ribonucleic acid (RNA) into the female oocyte, suggesting a possible role of sperm RNA during or after the fertilization process (Miller and Ostermeier, 2006; Piña-Aguilar, 2006; Gilbert et al., 2007; Hamatani, 2012). Some examples of sperm mRNA related to male fertility are: Aromatase (Lambard et al., 2003), Pregnancy-Specific-Beta-1-Glycoprotein (PSG1) (Avendaño et al., 2009), and Nitric Oxide Synthase (NOS) (Ishikawa et al., 2005). The expression of these genes mRNAs, have been associated with sperm motility, viability, progress movement and capacitation in various species but have not been studied in bulls. Therefore, the current investigation aimed to investigate the expression of the genes Aromatase, PSG1 and NOS in bull semen.

OBJECTIVES

- To determine the effect of breed, type of cattle, and age in the genetic expression (relative amounts of mRNA) of Aromatase, PSG1, and NOS in bull semen of dairy and beef bulls.

- To determine the correlation of the expression of the genes Aromatase, PSG1, and NOS with scrotal circumference.

Chapter 2. LITERATURE REVIEW

I. DAIRY AND BEEF PRODUCTION IN PUERTO RICO

Among the top 10 agriculture commodity groups of Puerto Rico, the higher contributors are dairy (mostly milk) and beef production (División de Estadísticas, 2011). Dairy is one of the main agricultural productions on the island, and contributes with a total gross income of $237,107,000 (División de Estadísticas, 2011). However, the dairy industry in Puerto Rico has been facing an economic crisis, accounting more than 645 million pounds of milk in losses, in 2011 (NASS, 2012). On the other hand, the beef industry, produced 17.2 million pounds of meat, but consumption was 156.8 million pounds (Casas, 2011).

Globally, the animal industry has been impacted by increases on feed cost, fuel, electricity and animal health supplies. Therefore, farm management is key to success in production. Reproduction and genetic selection are very important in farm management, thus obtaining animals with better traits of economic importance may improve production efficiency.

II. REPRODUCTION ON THE FARM

In the Tropics, one of the main concerns in both, dairy and beef cattle industry is problems with the reproductive performance. Successful reproduction of animals is fundamental in any cattle farm in order to improve genetics stocks and productivity.

Animal reproduction has been focused mainly on selection of fertility traits on the female to optimize fertility (Cammack et al., 2009). However, according to Mackinnon et al. (1990) using molecular biology techniques, cow fertility could be improved by the selection for fertility traits on the bull. The fertility and the role of the bull is extremely important in order to successfully mate on the farm. Nevertheless, in Puerto Rico the evaluation of a bull reproductive

condition is a practice that is often neglected by farmers. Many producers in Puerto Rico believe, incorrectly, that bulls with a high body score condition (BSC) and good phenotype will be good sires (Lunstra and Echternkamp, 1982; Bruner et al., 1995; Stowe et al., 2013). Farms with natural breeding programs often encounter low pregnancy rates (Cammack et al., 2009) perhaps due to factors associated to the bulls, for example; infertility, sperm abnormalities, or abnormal genitalia. Moreover, most sires in Puerto Rico are never given a breeding soundness examination (BSE). A BSE is recommended annually and consists of a physical examination and a semen sample to ensure that the animals are in optimal condition (Kastelic, 2014). The current cost of a BSE is usually under $50 and is considered a wise investment (Roberts, 2008).

Bulls with low fertility rates will decrease the amount of calves in the herd (Kastelic, 2014), and at the same time can be expensive for the farmer because they are not economically viable. If farmers have bulls that have been given a BSE and fertility analysis with outstanding results, automatically the bull and its semen will have more value. As a result, tested bulls will improve the genetic pool and could give an additional profit to the farmers.

III. BULL REPRODUCTIVE PERFORMANCE

Improvements in the reproductive performance of a dairy or beef cattle farms can be achieved through: genetics, nutritional plans, management improvement and environmental conditions (Spitzer and Hopkins, 1997). Additionally, the breed, age and scrotal circumference may influence the reproductive performance of the bull (Cammack et al., 2009).

Ellis et al. (2005) stated that the fertility potential can be affected by certain environmental condition, and can have impact over certain breeds and animal age. In Puerto Rico there are few varieties of bull breeds. For dairy cattle, the most common breed is Holstein

followed by Jersey and Brown Swiss. For beef cattle, the breed variety are much larger; Angus, Brahman, Brangus, Charolaise, Charbray, Senepol and Simmental, among others. According to Parkinson (2004), dairy bulls' reproductive management has been overlook compared to their female counterparts. Today, most dairy farms combine an artificial insemination (AI) program and the use of natural breeding for cattle that failed to achieve pregnancy. However, for beef cattle, natural service is still the most predominant practice (Parkinson, 2004). The selection of genetically superior bulls breeds for natural service or AI, will depend specifically on the purpose of production, and the reproductive goals of the farm (Lunstra and Echternkamp, 1982). The role of the bull on the farm will depend on the fertility potential. However, this potential will depend on factors like management, bull-cow ratio, the length period with cows and mating ability (Parkinson, 2004; Cammack et al., 2009).

Bull age has been directly related to the dominance and reproductive performance in the herd, more dominant (mature) bulls had greater serving capacity compared to young (immature) bulls (Makarechian and Farid, 1985; Barling et al., 1997; Lopez et al., 1997; Perry and Patterson, 2011). An investigation on the relative gene expression in rats, reported an age-related change with cellular localization of Aromatase activity, expressed in Sertoli cells on immature animals, while Leydig cells are in mature (Papadopoulos et al., 1986).

The age at which young sires reach puberty is crucial and could affect their fertility. Furthermore, it has been shown that puberty acceleration (e.g nutrition) can cause inadequate sperm maturation and thus lower fertility (Saacke and White, 1972; Wells et al., 1972; Jones, 1975). Ellis et al. (2005) stated that bulls between the ages of 12-24 months are in a persistent sexual and physical maturation state and any drastic change could jeopardize their fertility potential. Also, it has been suggested that, after young sires reach puberty (e.g. first four months)

a poor quality of semen sample can be expected (Lunstra and Echternkamp, 1982).

Bull scrotal circumference could be an indicator of the sperm output, and greater size is highly correlated with daily sperm output (Parkinson, 2004). Bulls used for natural breeding should have a minimum scrotal circumference of 30 cm (Spitzer and Hopkins, 1997). In addition, some investigations have associated scrotal circumference with motility, and increase sperm output with higher fertility in bulls (Kastelic, 2014).

Martínez-Velázquez et al. (2003) correlated the scrotal circumference with the age at puberty onset in heifer can be expected and reported that for every one-centimeter increase of a bull scrotal circumference there is a four-day decrease of heifers' age. A bull's scrotal circumference has been highly correlated with the age that a sire's daughter reaches puberty. This highly desirable reproductive trait was reported by Wilson (1977) and Morris et al. (2000) through two positive correlations (-0.30 and -0.25), respectively. Moreover, Makarechian and Farid (1985) were able to related scrotal circumference and the bull fertility.

IV. BULL FERTILITY

Fertility is the ability of the bull to produce a healthy offspring. In the field, the fertility potential of the bull can be evaluated by the mating ability, a physical examination and semen sample. A bull mating ability will be defined as the breeding potential and ability to complete fertilization process. But, in order to mate, a physical examination should be performed. The physical examination consists of the genitalia of the bull, that involves penis, prepuce, palpation of accessory glans, scrotum, scrotal circumference and semen sample. The quality of the bull semen sample is directly related to the physical characteristic of the genitalia. But, also the

fertility of the bull, will depend on the semen quality, and characteristic like volume, morphology, motility, and viability (Lalancette et al., 2008).

V. SPERM QUALITY

The sperm cell is a unique feature of the male reproductive system, sperm success depends on its quality and composition. Recent investigations proposed that the sperm quality depends on the spermatogenesis process, and it has been suggested that even some genes involved in this process are; Aromatase (Carreau et al., 2009; S. Carreau and C. Travert, 2010; S. Carreau and C. Travert, 2010; Said et al., 2014) , PSG1 (Avendaño et al., 2009; Martinez et al., 2013) and NOS (Adams et al.,1992; Rosselli et al., 1998).

Ostermeier et al. (2004) reported that the sperm contribute with its own transcripts produced through spermatogenesis, and there is no overlapping between the mRNA of the oocyte and the sperm. Just as it was discovered for these transcripts in spermatogenesis, the same happen for the present of PSG1. The presence of PSG1 was reported only in females, but now we know that the men sperm carry some PSG1 mRNA (Ostermeier et al., 2002; Zhao et al., 2006). Besides, in the men sperm, spermatogenesis has been established as the quality process of the sperm, and it have been suggested that Aromatase is essential in this process (Robertson et al., 1999).

During the sperm formation, it is exposed to a constantly changing environment, which ensures the maturation, acquisition of the potential fertilization and progressive motility (Pons-Rejraji et al., 2009). Also, the epididymis is the quality control for a healthy sperm, which packages the mature genome (Mewe et al., 2006). A mature and functional sperm is related to its

protamines mRNA (Miller et al., 2005). Also, this protamines may play a role in the formation of a functional sperm (Hamatani, 2012).

A. SPERM MORPHOLOGY

During spermatogenesis, Aromatase contribute in the sperm formation, and may play an important role in the acrosome formation (Robertson et al., 1999). Sperm function, is related to a normal acrosome formation, and significantly correlates with higher fertility (Saacke and White, 1972). However, abnormalities of the head of the sperm, like acrosomal defects can affect the fertility potential of the sperm (Parkinson, 2004).

The quality and morphology of the sperm, will depend of the spermatogenesis, and should produce a semen sample with high viability, motility and progress movement. These characteristics could be used as an indicator of the sperm quality and functional male reproductive system (Saacke, 1982; Aleisa, 2013).

B. MOTILITY

Motility could be used as an indicator of sperm fertility, a sperm need to be motile in order to complete the fertilization process. Also, motility is a way to evaluate if the epididymis and testis are working properly (Bissonnette et al., 2009).

Aromatase expression has been related to higher sperm motility in men (Ostermeier et al., 2004), mice (Robertson et al. 1999, 2001) and buffalo (Tiwari et al., 2008). Mice with Aromatase (aromp450) deficiency develop infertility do to inability to complete the spermatogenesis process. As this process is affected, a decrease in sperm motility can be expected. As a result, lower motility is related to inability to complete the fertilization process

(Carreau and Travert, 2010). Additionally, an inverse correlation of Aromatase expression with sperm motility has been reported with lesser Aromatase expression and greater abnormal and immotile sperm (Carreau et al., 2009). Different to Said et al. (2014) that correlated higher Aromatase expression to increased sperm pH and decreased sperm motility. However, Lambard et al. (2003), Tiwari et al. (2008) and Carreau et al. (2009) suggested that Aromatase could be a putative marker in the acquisition of sperm motility capacity.

On the one hand, there are some investigations that support the role of nitric oxide to improve motility (Hellstrom et al., 1994; Lewis et al., (1996), where others reported that nitric oxide can inhibit sperm motility by inhibition of cellular respiration, causing lower motility and fertility (Rosselli et al.,1995: Weinberg et al.,1995). On the other hand, the overexpression of NOS can cause oxidation of sperm membranes lipids and proteins (Stamler et al., 1992), reduction of motility (Balercia et al., 2004), which could be related to infertile men. Also, Lambard et al. (2004) reported that NOS were significantly higher in sperm from low motility compared to high motility. However, the positive or negative role of NOS in the sperm would depend on the expression level (Balercia et al., 2004).

C. VIABILITY

In mammals, to preserve sperm viability they are stored in the caudal epididymis of the testis (Jones, 2004). The viability of the sperm will depend on factors like temperature 37°C and collection time (Kaabi et al., 2003). Bertol et al. (2013), reported that sperm motility remains viable for up to 40.8 hours after collected through electroejaculation, if the sample is preserved at 5°C. The sperm viability could be affected no only by temperature and collection time, it would also depend on the gene expression in the sperm. According to (Galeraud-Denis et al., 2009)

sperm with high transcripts level of Aromatase is related to increased sperm pH, which would reduce the viability of the sperm.

D. CAPACITATION

The capacitation begins when the sperm is ejaculated, after this process the sperm undergo metabolic and structural changes. Once the sperm pass through this process it gains hyperactivated motility, bind the zona pellucida and undergo acrosomal reaction. During these process the sperm not only carry the paternal DNA, it also carry some mRNA remnants that help the sperm complete the fertilization process. Some investigation suggest that NOS mRNA is involve in the capacitation process and acrosomal reaction (Rodríguez et al., 2005). In fact, in bulls NOS is involved in the capacitation process, participating by intracellular mechanism (Rodríguez et al., 2005). Also, NOS is involved in the acrosome reaction, it help the sperm retain intact the acrosome to properly performed fertilization (Herrero et al., 1997). Different to Zini et al. (2005), that described that low concentration of NOS level increased capacitation in men sperm.

VI. SPERM POST-FERTILIZATION PERFORMANCE

Sperm RNA may play a role in the post-fertilization, contributing with a complete structure for fertilization, and embryonic development (Hamatani, 2012). In fact, it has been suggested that sperm could retain some PSG1 mRNA (Avendaño et al., 2009). This PSG1 mRNA works as an immune modulators during the fertilization process, and could be detected 24hr after fertilization (Hamatani, 2012). Also, it has been suggested that PSG1 have a role in

early embryogenesis (Lalancette et al., 2008) and implantation (Hamatani, 2012;Martinez et al. 2013), but the physiological pathways are not clear yet.

VII. SPERM RNA AND GENES EXPRESSION

Research in men sperm demonstrated that RNA is located in the nuclear periphery, specifically in the nuclear matrix (Pessot et al., 1989; Wykes et al., 1997; Dadoune et al., 2005), while, other suggested that is located on the mid-piece of the sperm tale (Hamatani, 2012, cited in (Kumar et al., 1993; Modi et al., 2005). It has been suggested that this RNA is ejected on the final step of spermatogenesis, leaving only the RNA remnants in the sperm. Contrary to this, Ostermeier et al. (2002) proposed that the sperm selectively retain some RNAs during spermatogenesis. Additionally, it has been reported that the sperm RNA remnants could potentially be used as a fertility marker and diagnostic tool for infertility, even if RNA is not functional (Mewe et al., 2004; Garrido et al., 2009; Hamatani, 2012). The identification of RNA in sperm may help to provide a better understanding of spermatogenesis and fertilization process (Lalancette et al., 2008).

The RT-PCR is a reliable technique and has the capability to identify the expression of mRNA for several genes in body fluids, including semen (Caron et al., 2001). Nevertheless, some investigators suggest that the mRNA is unstable and rapidly degraded and the detection is not suitable in biological samples (Sakurada et al., 2009). However, the messenger RNA (mRNA) could provide information about the sperm quality and at the same time be used as a tool for fertility detection (Lambard et al., 2003).

In spite of this sperm mRNA has been isolated in mice (Gyllensten et al., 1991), humans (Lambard et al., 2003; Zhao et al., 2006; Avendaño et al., 2009), buffalos (Tiwari et al., 2008),

pigs (Hwang et al., 2013) and chickens. By means of RT-PCR assay, semen mRNA can be used for many purpose, including detection of sperm quality, early affected sperm and even as a diagnostic tool for fertility (Krawetz, 2005).

The mRNA for the genes Aromatase, PGS1 and NOS have been detected in sperm from men and other species, and also have been related to fertility. Detection of these genes in bovine sperm could possibly be used as fertility markers in bulls, as it has been demonstrated in humans.

Aromatase is an enzyme responsible for the irreversible transformation of androgens into estrogens (Lambard et al., 2003). This transformation is crucial for a normal male sexual and reproductive development, which has been related to the Aromatase balance and male fertility (Saez, 1994; Carreau et al., 2003). Furthermore, mice deficient in Aromatase, are infertile, to disrupted spermatogenesis and inability to convert androgens in to estrogens (Robertson et al., 1999). Aromatase deficiency, is related to the inability to complete the fertilization process and if this process can not be completed lower pregnancy rate can be expected (Levallet et al., 1998; Robertson et al., 2001, 1999).

PSG1 is a major placental glycoprotein that may play a role in supporting early gestation and could modulate T-cell regulation (Motran et al., 2002). But, recently the expression of PSG1 has been reported in men sperm. Avendano et al. (2009) confirm the expression of PSG1, and his investigation revealed that sperm from fertile male, expresses higher quantity of PSG1 compared to non-fertile, and suggested a possible role on sperm during embryo development. Additionally, Human Leukocyte Antigen-E (HLA-E) and PSG1 expression levels were significantly higher in

samples from fertile men when compared to infertile, suggesting a possible association with both genes on the sperm and fertility potential (Avendaño et al., 2009).

Nitric Oxide Synthase (NOS) is an enzyme that might play a role in signal transduction of biological process (IG-Narro et al., 1987). NOS expression has been located in the acrosome and tail of the male and mouse sperm (Lewis et al., 1996). NOS, has a crucial role in the production of Nitric Oxide, which has been related to several men reproductive process, in particular, regulation of spermatogenesis, sperm motility and testicular function (Adams et al., 1992; Rosselli et al., 1998; Ishikawa et al., 2005). As NOS is involved in many men sperm process, it has been suggested as a fertility indicator (Lewis et al., 1996).

It has been shown that sperm mRNA for the genes Aromatase, PSG1 and NOS may play important roles in sperm function and fertility of men and other species (Rodriguez et al., 2005; Tiwari et al., 2008; Avendaño et al., 2009). Besides, these genes in the sperm could even play a role before or after fertilization process. In fact, there could be a possible function of sperm motility, progress movement and viability. To our knowledge, gene expression of the Aromatase, PGS1 and NOS in bull semen has not been investigated, therefore the current investigation aims to study the expression of the genes Aromatase, PGS1 and NOS in bovine semen.

Chapter 3. EXPRESSION OF PREGNANCY-SPECIFIC BETA-1-GLYCOPROTEIN, AROMATASE AND NITRIC OXIDE SYNTHASE IN BOVINE SPERM

INTRODUCTION

Reproduction is fundamental in efforts to improve animal production. Research to better reproductive performance by using genetic selection in bovines has been focused on optimizing fertility traits directly in the female (Cammack et al., 2009). However, the results of some investigations suggested that cow fertility could be improved by selecting for fertility traits in the bull (Mackinnon et al., 1990).

The sperm cell is a unique feature of the male reproductive system, which was typically thought to serve only to deliver its DNA for the fertilization process. Recently, various investigations have found that sperm metabolites may affect other reproductive functions. For example, the sperm can carry ribonucleic acid (RNA) into the oocyte, and it has been suggested that messenger ribonucleic acid (mRNA) might have a role before or after fertilization (Ostermeier et al., 2002; Krawetz, 2005). In addition sperm RNAs may contribute to subsequent processes such as early embryonic development and implantation (Hamatani, 2012).

In men, the genes that have been implicated with fertility includes: Pregnancy specific beta-1-glycoprotein (PSG1), Aromatase and Nitric Oxide Synthase (NOS). For example, sperm from fertile humans expresses a greater quantity of PSG1 compared to non-fertile individuals, (Avendaño et al., 2009). Also, it has been suggested that PSG1 may play a role in embryogenesis and in implantation (Ha et al., 2010). Furthermore, greater expression of the genes Aromatase resulted in higher sperm motility of men (Ostermeier et al., 2004) and buffalos (Tiwari et al., 2008). Thus, the degree of Aromatase expression in sperm was suggested as a possible marker for sperm motility (Lambard, 2004; Tiwari et al., 2008). Moreover, NOS is involved in the

regulation of spermatogenesis (Ishikawa et al., 2005) and could be involved in the processes of sperm maturation and capacitation (Rodríguez et al., 2005).

Collectively, these genes may play important roles in sperm fertility of men and other species. To our knowledge, gene expression from bull sperm has not been investigated, therefore the current investigation aimed to study the expression of the genes PGS1, Aromatase and NOS in bovine semen.

MATERIALS AND METHODS

A. GEOGRAPHICAL LOCATION

This investigation was conducted in Puerto Rican dairy and beef cattle herds, located in the following municipalities: Lajas, Cabo Rojo, Humacao, Guanica, Vega Alta, Camuy, Juana Díaz, Santa Isabel, San Germán, Moca, Hatillo, Arecibo, Morovis, Corozal, Toa Alta, Jayuya, Utuado, Ciales, and at the Beef Cattle Farm "Finca Montaña" of the University of Puerto Rico Mayagüez Campus. The semen collections took place from late May 2011 until mid June 2012.

B. SEMEN COLLECTION

One semen sample was collected from bulls pertaining to the following breeds: Brahman (*n*= 21), Brangus (*n*= 6), Charbray (*n*= 7), Charolais (*n*= 16), Simmental (*n*= 4), Senepol (*n*= 13), Holstein (*n*= 30) and Jersey (*n*= 5). Sample sizes for each breed depended on the farm availability. Those animals were 18 months of age or older. Before each collection, information on the animal was gathered, including: identification number, breed, scrotal circumference and age. Previous to the collection, a visual and physical examination of reproductive organs was

performed. Only bulls considered normal and healthy had their semen collected by electro-ejaculation. The fresh semen was visually evaluated for quality and blood, urine, dirt or pus contamination. Subsequently, the semen sample was observed under a microscopic for the presence of microorganisms. A minimum volume of 5.0 mL of semen per animal was then placed in sterilized tubes.

C. TOTAL RNA ISOLATION FROM SPERM SAMPLES

The freshly ejaculated semen was transferred into 15 mL centrifuge tubes. For total RNA isolation, RNAzol® RT reagent (Molecular Research Center, Inc., Cincinnati, OH) was used. For each 1.0mL of sample in the tube, 1.0 mL of RNAzol® was added (1:1 ratio) within a total volume of 10.0 mL. The samples were homogenized for one minute in the Polytron® Homogenizer and further procedures recommended by the manufacturer were followed. The final RNA pellet was diluted with 50 µl of molecular grade water and placed in 1.7 uL microcentrifuge tubes, which were immediately stored at -80°C. An Eppendorf BioPhotometer Plus® with Hellma® TrayCell adapter was used to measured the concentration of total RNA. To optimize the quality of the samples and eliminate DNA and RNase activity, the RNA was treated with Amplification Grade Deoxyribonuclease-1 (DNase1) (Sigma-Aldrich, Inc., 2010) according to the manufacturer's instructions.

D. cDNA SYNTHESIS AND REAL-TIME PCR

The reverse transcriptase reaction was performed with Quanta Biosciences qScript cDNA SuperMix® (Gaithersburg, MD) following the manufacturer's procedures for a total volume of

20μL and using 300 ng of total RNA. The cDNA synthesis was performed in Eppendorff 6321 AG thermocycler as follows: 5 minutes at 25°C, 40 minutes at 42°C, 5 minutes at 85°C, and then kept at 4°C. The real time polymerase chain reaction (RT-PCR) was performed in Eppendorf Realplex 22331 with Quanta Biosciences PerfeCta SYBR Green Fast Mix® (Gaithersburg, MD) according to manufacturer's procedures. The following conditions were imposed for the reaction: 95°C for 2 minutes, followed by 45 cycles at 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. Specific primers for bovine transcripts were obtained from previous publications (Table 1) and final concentrations for the forward and reverse were 900 μM.

Table 1. Primer sequence for amplification of Pregnancy Specific Beta-1-Glycoprotein, Aromatase, Nitric Oxide Synthase and Ribosomal Protein S9 (RSP9)

Gene	Accession No.	Forward Primer	Reverse Primer	Reference
Aromatase	NM-174305	5'CGA AGT TGT GCC TAT TGC CAG CAT3'	5'AGA GGA ACC TGC AGT GGG AAA TGA3'	Peruffo et al. (2011)
Pregnancy Specific Beta-1 Glycoprotein	NM-174411	5'TGG CCT TCT CAG AGT GCA TAG TCA3	5'ATC CTT GAT GTT TCT CAG CGG GTG3'	Szenci et al.(2011)
Nitric Oxide Synthase	NM-181037	5'AAA GCA ACC ATC CTG TAC GC3'	5'ATT CCC AAA GGT GCT GGT CA3'	Yoshida et al.(2006)
Ribosomal Protein S9 (Housekeeping gene)	DT860044	5′CCT CGA CCA AGA GCT GAAG'3	5′CCTCCAGACCTCAC GTTTGTTC3'	Janovick-Guretzky et al. (2007)

E. STATISTICAL ANALYSIS

Relative expression of the genes in semen was related to age, breed, and type of cattle (dairy or beef). In addition, scrotal circumference was correlated with expression of the genes. The statistical analysis was performed using Statistical Analysis System (SAS) software (Version 9.1, SAS Institute Inc., Cary, NC, USA).

$$Y_{ij} = \mu + \alpha_i + \beta x_{ij} + \varepsilon_{ij}$$

Y_{ij} = relative gene expression (PGS-1, Aromatase,)

μ = overall mean

α_i = effect of the age [(18-28), (29-36), (37-60) and (61-120) months]; effect of type (dairy or beef cattle); or effect of breed (Jersey, Holstein, Brahman, Brangus, Charolais, Charbray, Senepol and Simmental);

βx_{ij} = covariate effect, level of expression of RPS9

εi_j = experimental error associated with total or relative expression RPS9

SCROTAL CIRCUMFERENCE

Thirty-one bulls were restrained and scrotal circumference was measured with a scrotal wand. A simple linear regression between relative gene expression and the housekeeping gene RSP9 was calculated. The PROC REG procedure of SAS was used to determine the residual values.

PEARSON CORRELATION COEFFICIENT

The residuals values were used for this purpose. The PROC CORR procedure of SAS was used to calculate the correlation coefficient between scrotal circumference and relative gene expression.

RESULTS AND DISCUSSION

In this investigation, the mRNA for the genes PGS1, Aromatase, and NOS in bovine semen was detected. In addition, the effects of bull breed (Figure 1.0, 1.1, 1.2), type of cattle (Figure 2.0, 2.1, 2.2) and a correlation with scrotal circumference were determined (Table 2). Meanwhile, no significant effect of age group was observed (Figure 3.0).

A.BULL BREEDS AND RELATIVE GENE EXPRESSION

The relative expression of Aromatase in sperm was lower for Jersey bulls compared to the other breeds [(1.01); $P<0.07$, Figure 1). However, the relative expression of PGS1 ($P = 0.45$) and NOS ($P = 0.11$) was similar among all breeds tested. Relative expression of PSG1 was 1.00, 1.03, 0.993, 1.15, 0.977, 1.015, 0.97, and 0.920 for Brahman, Brangus, Charbray, Charolais, Simmental, Holstein, Jersey, respectively. Corresponding values for NOS were: 1.00, 1.02, 0.96, 1.00, 0.88, 0.93, 0.95, and 0.83.

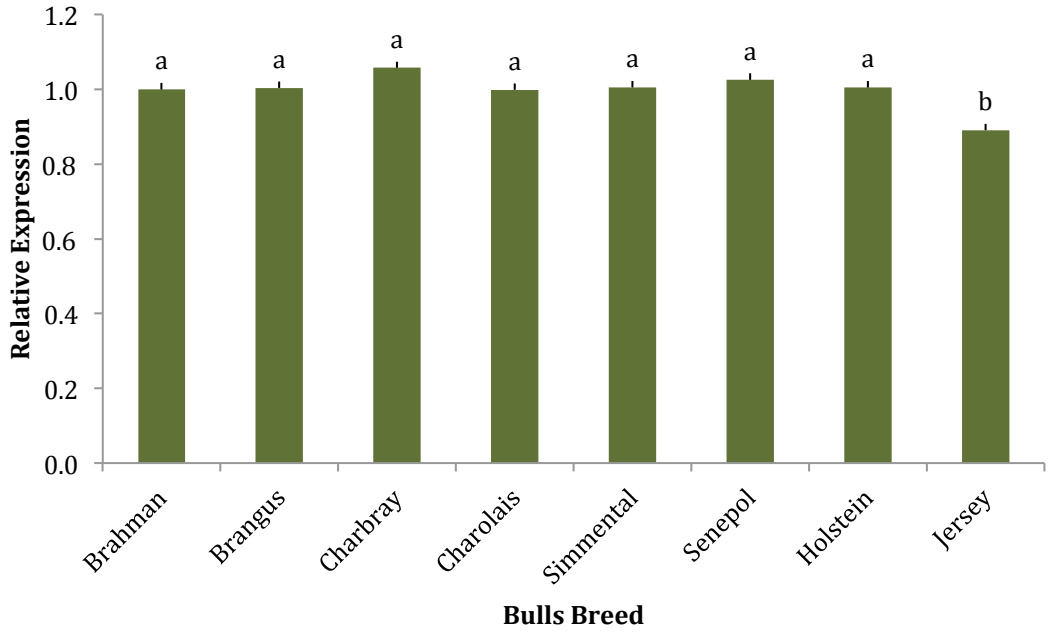

Figure 1.0 Relative Aromatase expression in eight bull breeds
Different letters represents significant differences at a level of (P< 0. 07).

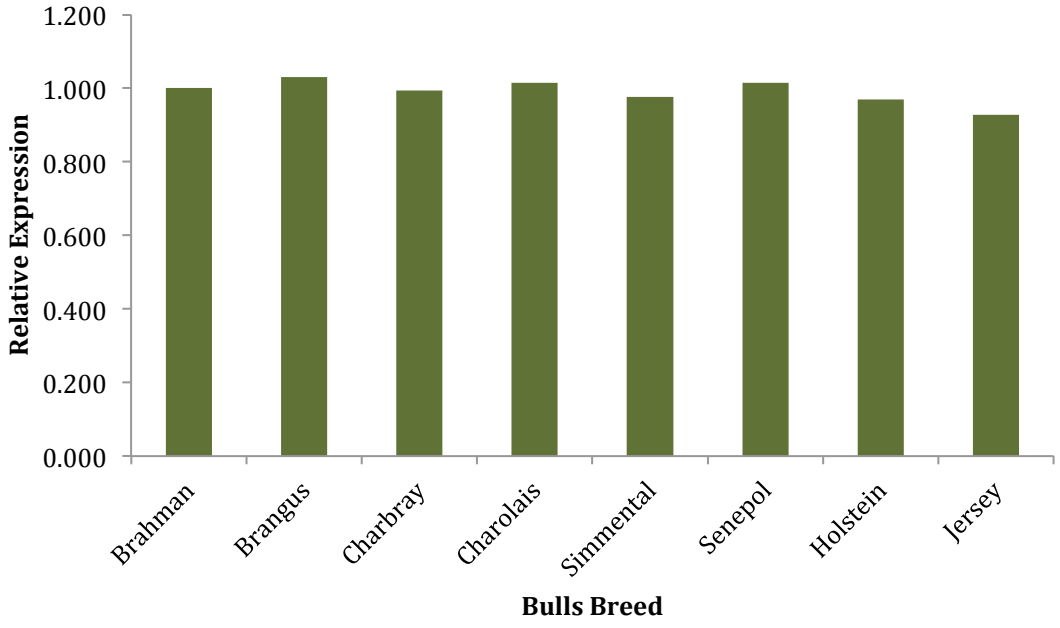

Figure 1.1 Relative Pregnancy-Specific-Beta-1-Glycoprotein expression in eight bull breeds
Relative expression of NOS was similar among breeds (P = 0.45).

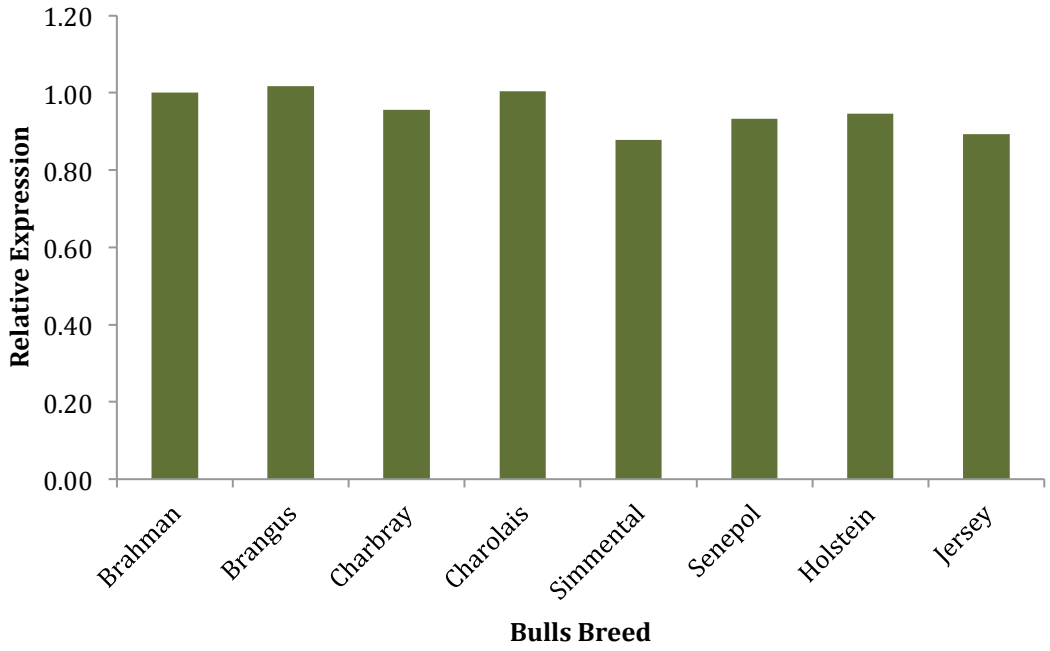

Figure 1.2 Relative Nitric Oxide Synthase expression in eight bull breeds
Relative expression of NOS was similar among breeds (P = 0.11).

B. TYPE OF CATTLE AND RELATIVE GENE EXPRESSION

The relative expression of aromatase (1.00 beef versus 0.98 dairy) did not differ significantly between the two types (P=0.18), whereas the relative expression of PSG1 in sperm was lower (P<0.02) for beef (0.96) than for dairy cattle (1.00), NOS showed the same relative expression of 1.00 in both beef and dairy cattle (P=0.90). The relative expression of the genes can be found (Figure 2.0, Figure 2.1, and Figure 2.2, respectively).

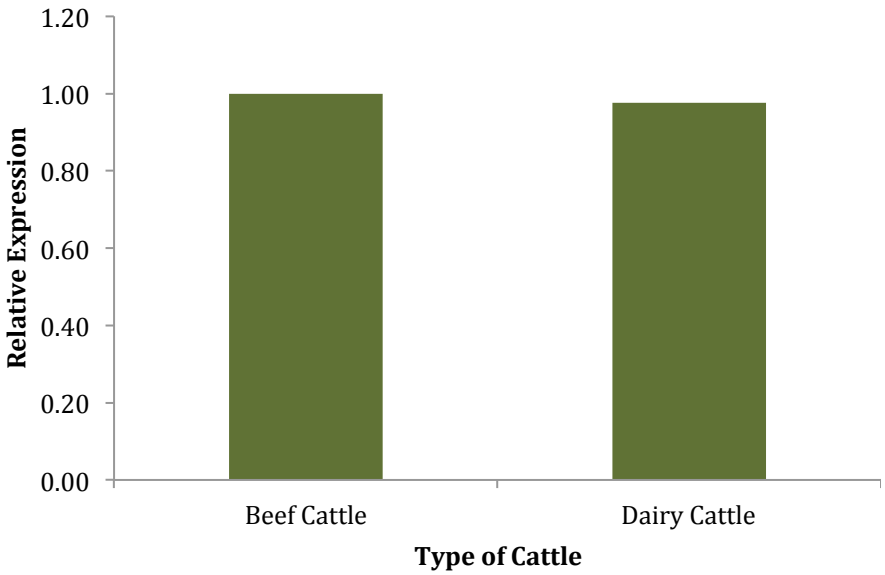

Figure 2.0 Effect of Type of Cattle on Relative Expression of Aromatase
Relative expression of Aromatase was similar among type of cattle (P = 0.18).

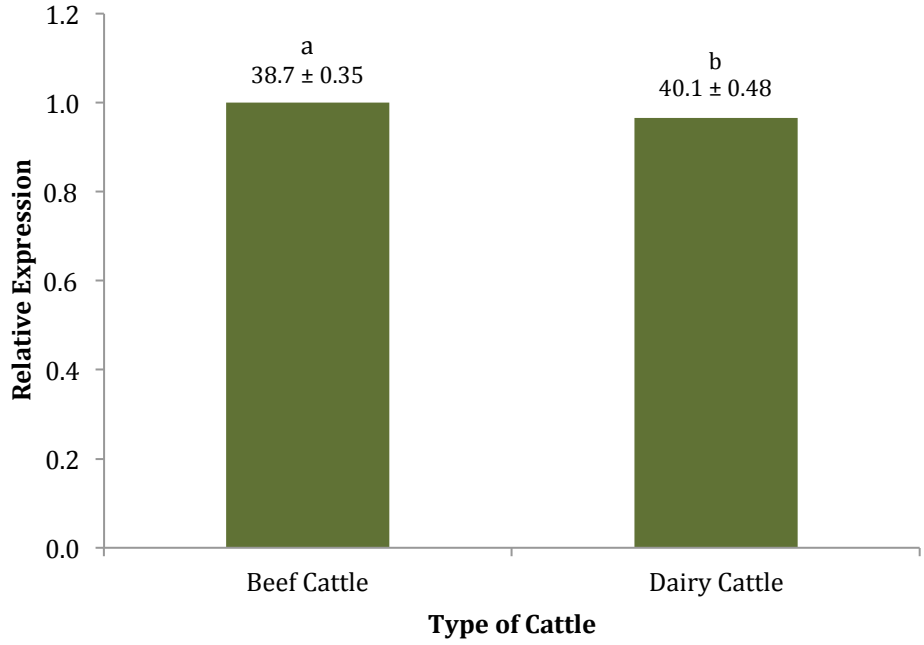

Figure 2.1 Effect of Type Cattle on Relative Expression of Pregnancy-Specific Beta-1-Glycoprotein
Different letters represents significant differences at a level of (P< 0. 02).

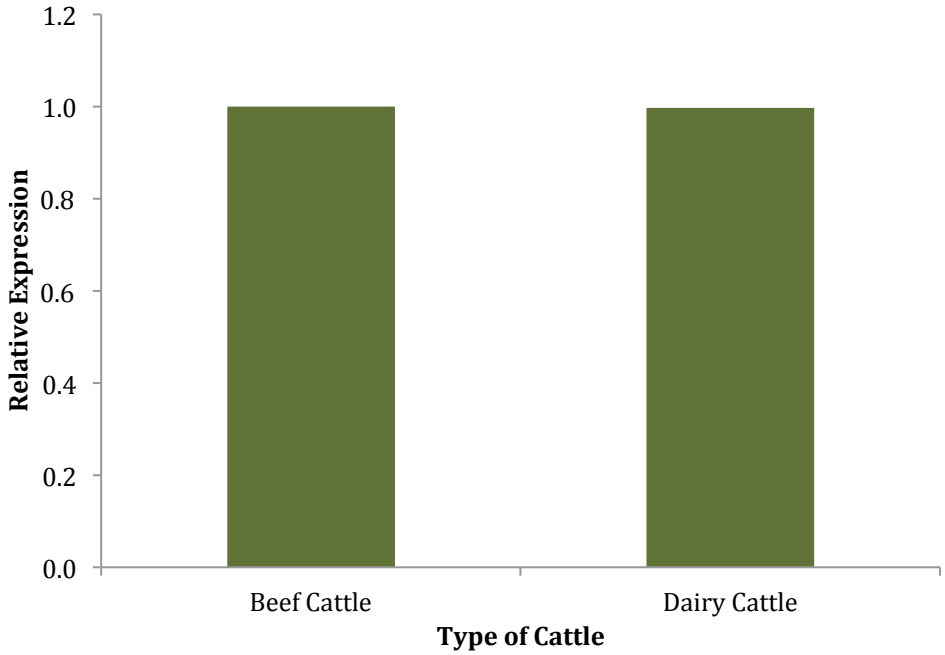

Figure 2.2 Effect of Type of Cattle on Relative Expression of Nitric Oxide Synthase
Relative expression of ONS was similar among type of cattle (P = 0.90).

C. BULL AGE AND RELATIVE GENE EXPRESSION

Significant differences among age groups were not found in expression of aromatase (P = 0.23), PGS1 (P = 0.39), and NOS (P = 0.89) for the age categories 18-24, 25-36, 37-60, 61-120 months. The respective qRT-PCR values observed for each category were: Aromatase, 1.00, 1.05, 1.04, 1.031; PGS1, 1.00, 0.97, 0.97, 0.96; NOS, 1.00,1.00,1.01,1.03 (Figure 3.0).

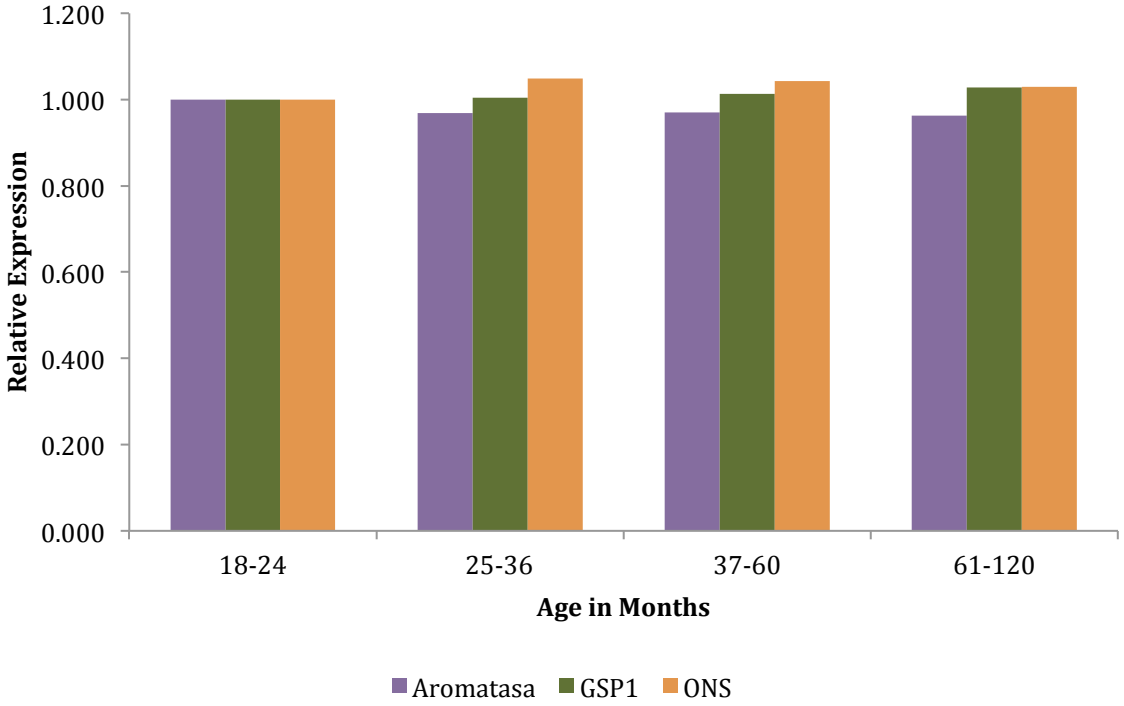

Figure 3.0 Effect of Bull Age on Relative Expression of Aromatase, Pregnancy-Specific Beta-1-Glycoprotein and Nitric Oxide Synthase
Relative expression of was similar among age categories.

D. SCROTAL CIRCUMFERENCE

The correlation analysis (n=31) showed that NOS had a positive association (P<0.08) with scrotal circumference but the relative expression of aromatase and PGS1 were not significantly correlated with this anatomical and reproductive trait (Table 2).

Table 2. Pearson correlation coefficient between PSG1, Aromatase, NOS mRNA expression and scrotal circumference in dairy and beef bulls.

Gene	Correlation	P-value
Aromatase	0.1716	0.3559
Pregnancy-Specific Beta-1-Glycoprotein	-0.1966	0.2892
Nitric Oxide Synthase	0.3149	0.0844

The sample corresponded to a total on thirty-one dairy and beef bulls.

Nussbaumer et al. (2006) and Sakurada et al. (2009) stated that the RT-PCR technique is a reliable tool for the identification of semen mRNA expression. However, the mRNA is unstable and can be degraded rapidly (Sakurada et al., 2009). In spite of this, sperm mRNA has been isolated in mice (Gyllensten et al., 1991), humans (Lambard et al., 2003; Zhao et al., 2006; Avendaño et al., 2009), buffalos (Tiwari et al., 2008), pigs (Hwang et al., 2013) and chickens (Shafeeque et al., 2014). Therefore, the RT-PCR assay was used in the present study to evaluate the mRNA expression for Aromatase, PSG1 and NOS in bulls.

Aromatase has been established as an important player in the male reproductive system, and "normal" male reproduction and sexual development has been related to the balance of its activity (Saez, 1994; Carreau et al., 2003). In fact, aromatase expression has been positively related to higher sperm motility in men (Ostermeier et al., 2004), mice (Robertson et al., 1999, 2001) and buffalo (Tiwari et al., 2008). In that sense, earlier investigations suggested that aromatase expression could be used as a possible marker for sperm motility (Lambard, 2004; Tiwari et al., 2008; Carreau et al., 2009). The Jersey bulls of the present study exhibited lesser

aromatase expression than the other breeds sampled, which differs from the results of previous investigations (Tiwari et al., 2008). No previous scientific support the relation of lesser aromatase expression on the Jersey breed. However, aromatase deficiency is related to inability to complete the fertilization process and lesser expression of this gene has been negatively correlated with greater proportions of abnormal and immotile sperm (Carreau et al., 2009).

The presence of PSG1 was reported for the first time in male sperm by Avendaño et al. (2009) and confirmed by Hamatani (2012). They concluded that sperm from fertile men show greater PSG1 expression than sperm from non-fertile men. Our results demonstrated an effect of animal type on the expression of PSG1 in bull semen, being greater for beef ($P=0.02$) compared to dairy cattle. Based on this result and previous investigations, we can postulate a possible relation between the PSG1 expression and greater fertility in beef than in dairy cattle. Results obtained in humans (Avendaño et al. 2009) and mice (Robertson et al., 1999, 2001) support this hypothesis. It has been suggested that sperm could retain some PSG1 mRNA (Avendaño et al., 2009) and play a possible role after fertilization, such as in early embryogenesis and implantation (Hamatani, 2012; Martinez et al., 2013), but the physiological pathways involved (Martinez et al., 2013) are not clear yet.

Avendaño et al. (2009) reported an age-related pattern of aromatase relative gene expression. However, the present results revealed no such effect of the age groups studied.

The expression of NOS is also related to male sexual and reproductive functions and sperm motility. Lewis et al. (1996) even suggested that sperm contain NOS transcripts located in the head and mid-piece. Lewis et al. (2006) reported a high correlation between NOS expression and testicular function. NOS expression was detected in bull semen in the present study, and a positive correlation with scrotal circumference was revealed ($r=0.31$, $P<0.08$). The present and

previous investigations provides evidence of a possible relation between NOS expression, testicular function and sperm motility. Some experimental results support a NOS role in the sperm motility (Hellstrom et al., 1994; Ishikawa et al., 2005), whereas others differ (Rosselli et al., 1995; Weinberg et al., 1995). On the other hand, the overexpression of NOS can cause oxidation of sperm membranes lipids and proteins (Stamler et al., 1992), reduced motility (Balercia et al., 2004), and other negative effects on infertile mens. Thus, the role of NOS will depend on the level of expression and redox state (Balercia et al., 2004).

These transcripts may have important roles in sperm fertility of men and other species. To our knowledge, the expression of these genes had not been previously investigated in bull semen. Therefore, our results document for the first time the presence of Aromatase, PGS1 and NOS transcripts in the semen of this species, with potential implications in sperm motility, fertility, embryogenesis, implantation and testicular function. These could potentially served as a diagnostic tool for bovine male fertility, which warrants further evaluation.

IMPLICATIONS

The expression of the genes aromatase, PGS2 and NOS was demonstrated in this investigation. A possible future application on bovine sperm mRNA could be as a diagnostic tool for fertility. Detection of these genes in bovine sperm mRNA may be used fertility markers in bulls, if further investigations correlate this expression with sperm quality. A transcriptome profiling could be used to identify if the genes Aromatase, PGS1 and NOS can be used as a marker for fertility for bull fertility. If these genes proved a high relation as fertility markers, they could be included as part of the BSE as a molecular examination. The expression of these genes in bull semen could potentially become a molecular marker for fertility as it has in humans.

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