# Effects of culture media on the *in vitro* maturation rate of bovine oocytes

by

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**Abstract**

Puerto Rico is located in a tropical zone characterized by a relatively constant high heat and humidity. As a result, cattle are under moderate to severe thermal stress on a year-round basis, depending on the time of the year. Thus, the problem of thermal stress can be considered chronic and one of the net results is a decrease in reproductive efficiency. The objective of this experiment was to study the feasibility and thermo- tolerance of *in vitro* matured oocytes collected in different seasons. For this, 87 dairy-breed ovaries (Holstein) and 120 beef-breed ovaries (Senepol, Zebu and cross breeds) were obtained in abattoirs Ganaderos Santiago and Hermanos Alvarado during four seasons [December-February (S1; 20°C - 29°C), March and April (S2; 20°C - 30°C), June to August (S3; 22°C - 32°C) and September to November (S4; 22°C - 31°C)]. A total of 804 oocytes were collected from 2-8 mm follicles. These were placed into three maturation mediums with different concentrations of Pluset (FSH y LH) and Estradiol (E2) (A: Pluset 5µg/µl and Estradiol 25µM; B: Pluset 25µg/µl and Estradiol 25µM; C: Pluset 5µg/µl) and matured *in vitro* for 22 hrs. in an incubator under a 5% $CO_2$ environment at 37.5°C. The oocytes in medium A (lower concentration of LH, FSH and presence of E2) had a 73% maturation than those in medium B and C [59% average maturity; P <0.05]. Oocytes from beef type cows had better maturation rates when compared with those from Holstein cows during S4 [80% (beef) vs. 60% (dairy); P <0.05]. Beef cattle also demonstrated better maturation rates in S4 [80%] than dairy cattle in S3 [50%]. In turn, oocyte maturation from beef type cattle showed an increase in maturation during S2 [55%] to S3 [68%] and again in S4 [80%] while dairy cattle drastically reduced 21% in maturation from S2 [71%] to S3 [50%]; (P <0.05). These

results suggest that beef cattle produce oocytes better adapted to tropical conditions and have better maturation rates in culture mediums with low gonadotropins concentrations and high concentrations of E2.

## Resumen

Puerto Rico está ubicado en una zona tropical, caracterizada por un clima húmedo y de constante calor. Como resultado, el ganado bovino está todo el año bajo condiciones de estrés termal moderado a severo, según la temporada. De este modo, el problema de estrés termal puede considerarse crónico, donde uno de los resultados netos es una reducción en la eficiencia reproductiva. El objetivo de este experimento fue estudiar la viabilidad y termo-tolerancia de ovocitos madurados *in vitro* colectados durante diferentes épocas. Se colectaron 87 ovarios de vacas lecheras (Holstein) y 120 ovarios de vacas tipo carne (Senepol, Cebú y Cebú x Senepol) obtenidos en los macelos Ganadería Santiago y Hermanos Alvarado durante las cuatro épocas del año [diciembre a febrero (S1; 20°C - 29°C), marzo a abril (S2; 20°C - 30°C), junio a julio (S3; 22°C - 32°C) y septiembre a noviembre (S4; 22°C - 31°C)]. Se colectaron un total de 804 ovocitos provenientes de folículos de 2–8 mm. Estos fueron colocados en tres medios de maduración con diferentes concentraciones de Pluset (FSH y LH) y Estradiol (E2) (A: Pluset 5µg/µl y E2 25µM; B: Pluset 25µg/µl y E2 25µM; C: Pluset 5µg/µl) y madurados *in vitro* durante 22 hrs. en una incubadora bajo un ambiente con 5% $CO_2$. Los ovocitos en medio A (menor concentración de Pluset y presencia de E2) presentaron un 73% de maduración vs. aquellos en medio B y C [59% maduración promedio; $P<0.05$]. Los ovocitos provenientes de vacas tipo carne tuvieron mejores tasas de maduración cuando se compararon con aquellos provenientes de vacas Holstein durante S4 [80% (carne) vs. 60% (leche); ($P<0.05$). Las vacas tipo carne también demuestran mejores tasas de maduración en S4 [80%] que las vacas lecheras en S3 [50%]. A su vez, los ovocitos de vacas tipo carne demostraron un aumento en maduración durante S2 [55%] a S3 [68%] y nuevamente en S4 [80%] mientras que las

vacas lecheras redujeron su maduración drásticamente un 21% de S2 [71%] a S3 [50%]; ($P < 0.05$). Estos resultados sugieren que vacas de carne producen ovocitos con mayor termotolerancia cuales tienen mejores adaptaciones a las condiciones tropicales y tienen mejores tasas de maduración en un medio bajo en gonadotropinas y presencia de E2.

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# 1 Introduction

According to agricultural statistics, Puerto Rico's dairy industry is currently the number one agricultural industry on the island (Department of Agriculture, 2010). It contributes with approximately 23% of the total value of the gross farm income (GFI) providing approximately $214 million to Puerto Rico's economy (Departament of Agriculture, 2015). On the other hand, the beef industry provides 3.14% of the GFI with approximately $29 million (Departament of Agriculture, 2015). Over the course of the years, eventhough the dairy industry is still the top agricultural industry it has greatly reduced its contribution (i.e. 32.1% GFI/ approximately $230 million in 2011-2012 fiscal year; Departament of Agriculture, 2015). In order to maintain and/or improve this income, an efficient reproduction of dairy cattle has to be the leading management practice in the herds. However, climate changes and increases in feed costs, are seriously affecting the efficiency of reproduction of these animals by increasing heat stress and reducing proper nutrient provision.

Fertility is defined as the capability of animals to become pregnant and carry a pregnancy if served at the proper time of ovulation (Darwash et al., 1997; Pryce et al., 2004). If there is interference in the proper detection of estrus, ovulation, or cycling and/or if the maintenance of the embryo or fetus is not permitted, then fertility is compromised and leads to the failure of a full-term pregnancy (Pryce et al., 2004) which, in turn, will reduce production. Internal and external factors reducing fertility of cattle include nutrition, genetics (Butler, 2000; Lucy, 2001; Beever, 2006; Walsh et al., 2011) and heat stress (Hansen et al., 2001; Rivera and Hansen, 2001; Rensis and Scaramuzzi, 2003). Although cross breeding of cattle (*Bos taurus x Bos indicus*) is

helpful for thermo tolerance (Hansen, 2004), it is not enough to keep reproduction of these animals at their most efficient rate.

An alternative for an efficient reproduction of cattle in sub- tropical climates would be to produce embryos *in vitro.* This would aid in bypassing factors that adversely affect the reproductive efficiency of the cattle (Rensis and Scaramuzzi, 2003) and provide producers with a different strategyof serving their cows. For this process a better procedure of in vitro maturation needs to be studied (Salhab et al., 2011) in order to fit the needs and criteria of cattle raised in tropical zone.

## 2 Objectives

2.1 To determine the influence of culture media on the of in vitro maturation rate of bovine oocytes.

2.2 To compare bovine oocyte *in vitro* maturation collected from beef and dairy cattle.

# 3 Review of Literature

## 3.1 Oogenesis and Ovulation

To have a better understanding of how external and internal factors affect the reproduction and fertility of animals, particularly bovine, there is a need to have a good understanding of oogenesis and ovulation. In bovines and other mammals, meiosis begins in the germ cells during fetal development (Bilodeau-Goeseels, 2006). During this development, profase I takes place in which leptotene, zygotene and pachytene occur with the condensing and aligning of chromosomes and crossing-over, respectively (Smiljaković and Tomek, 2006). DNA transcription occurs in diplotene and (Smiljaković and Tomek, 2006) at this time the first meiotic arrest takes place until ovulation is stimulated by hormonal surges with the onset of puberty (Bilodeau-Goeseels, 2006).

When the female has sexually matured, the gonadotropins follicular stimulating hormone (FSH) and luteinizing hormone (LH) being secreted from the adenohypophysis (Beker-van Woudenberg et al., 2004; Sirard et al., 2007) now have the amount of receptors needed in the ovaries to begin estrus cyles. FSH favors follicular growth by stimulating somatic cells around the oocyte, thereby completing nuclear maturation (Sirard et al., 2007). As FSH and LH are secreted, the first arrest stage of the oocyte ceases and diakinesis starts, thus resuming meiosis (Beker-van Woudenberg et al., 2004; Smiljaković and Tomek, 2006). Smiljaković and Tomek (2006) describe that at the end of RNA synthesis, the germinal vesicle breaks down allowing the chromosomes line up and divide during metaphase I and anaphase I. Telophase I then follows by forming two nuclei, one of which becomes the first polar body and is expelled. After a

short interphase takes place, meiosis II begins with prophase II, breaking down the nuclear envelope and allowing the chromatids to align once again for metaphase II. Metaphase II is also known as the second meiotic arrest and occurs at the time when ovulation or estrus takes place (Bilodeau-Goeseels, 2006).

Ovulation of the oocyte also indicates that the cumulus-oocyte complex (COC) has expanded in order to aid spermatozoa in fertilization (Sirard et al., 2007). If fertilization occurs, then the second meiotic arrest would end (Bilodeau-Goeseels, 2006; Smiljaković and Tomek, 2006) and anaphase II would take place by division of the chromatids and, like in the first arrest stage, two nuclei will form expelling one as the second polar body during telophase II (Smiljaković and Tomek, 2006). The fertilized ovum would then continue the process of becoming an embryo and implant itself in the uterine wall for fetal development.

### 3.2 Nutrition

One of the factors that greatly contributes to the loss in bovine fertility, directly and indirectly, is nutrition (Robinson et al., 2006). A nutrient imbalance or inadequate diets for high production directly affects the individual's reproduction (Lucy, 2001; Pryce et al., 2004). Not only does it directly affect oocytes, ovulation, fertilization, embryo development and pregnancy, but it also affects hormonal circulation and nutrient-sensitive metabolites that influence the reproduction process (Robinson et al., 2006).

### 3.2.1 Nutritional effects on reproductive efficiency

Good reproductive efficiency depends on the optimum feeding of the cows, whose nutrient requirements vary with their physical state and nutritional needs in order to prevent metabolic disorders, specifically during peri-parturient periods, (Boland et al., 2001; Roche, 2006). Nutritional requirements change drastically at parturition as milk production increases, causing negative energy balance (NEB) whose severity and duration relates to dry matter intake and body conditioning (BC) at calving (Butler, 2000; Beever, 2006). Overfeeding proteins can worsen BC loss in early lactation (Butler, 2000; Beever, 2006) causing a more severe NEB resulting in more adipose tissue mobilization and collecting greater amounts of triacylglycerols in the liver (Butler, 2000). This, in turn, will lead to a great amount of non-esterified fatty acids (NEFA) that would no longer be oxidized into very low density lipoproteins (VLDL) by the liver causing further complications such as an increased production of ketone bodies (Beever, 2006). Yet, low amounts of protein intake will also have a negative effect on the digestion and milk production of the animal by not being able to efficiently utilize the feed intake (Beever, 2006). Great loss in BC reduces conception rates along with greater risks of low fertility and longer intervals to the first ovulation after parturition as seen further ahead (Butler, 2000). Studies performed between 1991 and 1998 found that a NEB, caused a decline in first service conception rate from 60% to 54%, an increase in calving intervals from 386 to 396 days, and an increase in cows with abnormal cycles from 13% to 26% (Mee et al., 2004; Beever, 2006). As can be observed, the effect of NEB affects detrimentally reproduction and in turn decreases fertility (Roche, 2006).

### 3.2.2 Nutritional effects on oocytes and ovulation

A strong connection has been observed between postpartum NEB during early lactation and the resumption of ovulation postpartum (Boland et al., 2001). Ovulation may not occur in animals on low dietary intakes but follicle growth and atresia will occur due to the effect on follicles and to poor BC (Boland et al., 2001). This may be caused by excess rumen degradable proteins which have been proven to have an adverse effect on oocytes by minimizing growth and the metabolism of the oocytes granulosa cells by the alteration of the biochemical environment of the follicle (Robinson et al., 2006). This means that the length of the calving to first ovulation interval, and calving to conception interval will be prolonged (Boland et al., 2001). Anestrus in bovines caused by NEB from feed intake restrictions were reported caused by insufficient circulating LH preventing follicle growth and ovulation, yet the concentrations of FSH do not seem to be altered by NEB (Boland et al., 2001). On the other hand, NEB also affects folliculogenesis by increasing concentrations of growth hormones (GH) but lowering the amount of insulin and GH receptors in the liver (Pryce et al., 2004). This causes a lower production of insulin-like growth factors (IGF) and binding proteins minimizing concentrations of IGF in circulation (Pryce et al., 2004). In turn, since low amount of insulin concentrations have an effect on initiating estrus postpartum causing a delay in estrus (Pryce et al., 2004).

Ovulation is also affected by inadequate nutrient consumption by altering the follicular wave and ovulation rate (Robinson et al., 2006). Alterations may be related to the cell entry rate of glucose in animals if given high energy and protein supplements (Boland et al., 2001). But, if the amount of glucose is not properly administered, then

the growing primordial follicles that emerge for maturation are affected by poor nutrition reducing the amount of follicles preparing for follicle growth and ovulation (Boland et al., 2001; Robinson et al., 2006). Ovulatory responses can be controlled depending on the type of diet and end products after digestion to improve ovarian function (Robinson et al., 2006).

### 3.2.3 Nutritional effects on embryo development and survival

Embryonic development and survival is also affected by nutrition when providing essential nutrients in histotroph production [oviductal and uterine secretions](Robinson et al., 2006). Histotrophs are indirectly affected by hormones such as progesterone and growth factors [e.g. IGF-1](Robinson et al., 2006). As wasmentioned before, poor nutrition with high protein intake has a negative effect on IGF and its binding proteins (Pryce et al., 2004). High protein intake can also compromise embryo survival by suppressing progesterone concentrations that directly affect embryonic development through the enhancement and increased production of the trophoblastic antiluteolysin, interferon *tau* (Robinson et al., 2006). Animals that are undernourished present a decrease in interferon *tau* and an increase in prostaglandin F2α (PGF2α) in endometrial tissues (Boland et al., 2001) that could lead to abortion of the embryo. However, the use of dietary polyunsaturated fatty acids (PUFA) can decrease the release of PGF2α improving embryo survival, if used properly (Robinson et al., 2006). Linoleic acid is a PUFA but using it as supplementation will reduce luteal progesterone concentrations

compromising embryo survival regardless of dominant follicles and high IGF-1 concentrations (Robinson et al., 2006).

Embryo development and survival is also affected by deficiencies and excesses of micronutrients (Robinson et al., 2006). Vitamins and trace elements generally affect embryo proliferation and differentiation by stimulating the synthesis of steroidal hormone synthesis, growth factor expressions and gene transcriptions (Robinson et al., 2006). Inadequate concentrations of micronutrients such as Vitamin E and Selenium an improvement of *in vitro* blastocysts production has been found, yet deficiencies in Vitamin A and Cobalt can lead to impaired embryo growth and even abortion (Robinson et al., 2006). Excess quantities of Vitamin A also affect embryo development adversely causing impediments in the development of the embryo's nervous system (Robinson et al., 2006).

As already described, nutrition is very important and ruminants need more than just essential nutrients due to their diversified digestion characteristics (Robinson et al., 2006). Imbalances in their diets can have detrimental effects on their reproductive efficiency as well as embryo development (Pryce et al., 2004; Robinson et al., 2006; Roche, 2006).

### 3.3 Genetics

The genetic makeup of bovines is also key in their reproduction. Females with high genetic milk yield potential have a reproductive performance that is inversely proportional to their production (Butler, 2000; Lucy, 2001, 2003; Pryce et al., 2004;

(Paula-Lopes et al., 2012). Yet, this effect is also increased due to the genetic selection for milk production (Lucy, 2003). Over the course of time this type of selection has led to a reduction of approximately 1% of first service pregnancy rates per year (Pryce et al., 2004). In order to increase early lactation, cattle have been modified to mobilize adipose tissue and have a higher level of nutrient breakdown (Lucy, 2003) which in turn causes for longer calving intervals, days to first service, and conception rates (Pryce et al., 2004). Due to this, cattle that is selected for higher milk production tend to have a larger negative energy balance, thus affecting reproduction (Butler, 2000; Pryce et al., 2004; Roche, 2006) as was previously described.

On the positive side, the selection of genes is also helpful. Some breeds, like those that are *Bos taurus indicus* have a better tolerance for hyperthermia, which positively affects the reproduction efficiency of cattle (Hansen et al., 2001; Hansen, 2004; Ashrafzadeh et al., 2013). These breeds have a better system of thermoregulation (Brito et al., 2003; Paula-Lopes et al., 2012; Ashrafzadeh et al., 2013). As further described, these animals have genotypes encoding for phenotypes that provide larger sweat glands and sleek hair (Hansen, 2004). Not only does this affect *Bos taurus indicus* but there are some new world *Bos taurus taurus* breeds, such as Senepol, that have these characteristics as well (Hansen, 2004). Having this short and shiny hair, allows the animal to reflect the sun's radiation inducing a reduction in body temperature (Brito et al., 2003; Hansen, 2004; Ashrafzadeh et al., 2013). As the body temperature reduces, animals are better able to use their metabolic energy for reproduction instead of heat dissipation (West, 2003).

Genetics also contributes to the reproduction process at a cellular level. It has been found that cattle such as *B. indicus* and Senepol have acquired genetic protection to elevated temperatures (Hansen, 2004) by: 1) having lymphocytes that are more resistant to apoptosis induced by heat (Kamwanja et al., 1994; Paula-Lopes et al., 2003); 2) embryos that demonstrate further development in elevated temperatures (Paula-Lopes et al., 2003) and 3) by better endometrial responses (Malayer and Hansen, 1990). The amount of heat shock protein 70 (HSP70) in these lymphocytes could very well be a factor but there is no difference of HSP70 quantities between animals that are thermo-tolerant (Brahman and Senepol) vs non thermo-tolerant (Holstein) (Kamwanja et al., 1994).

## 3.4 Heat Stress

### 3.4.1 Effects of heat stress on fertility and conception

According to Ewel and Whitmore (1973), Puerto Rico is subtropical because sea level mean biotemperatures are lower than 24°C, but mean temperatures do reach much higher levels. As it is known, one of the major limitations affecting the pregnancy of dairy cattle in Puerto Rico is heat stress. Hyperthermia not only affects reproductive performance but also milk production. Heat stress is associated with a decrease in dry matter intake and energy balance to dissipate body heat (West, 2003). These negative effects may limit the energy available for production resulting in a metabolic disorder that may cause a late or a non-visible estrus (Kornmatitsuk et al. 2008; Hansen, 2009). As the temperature humidity index rises from 68, cattle are affected adversely

(Zimbelman et al., 2009) due to increases in rectal temperatures. When rectal temperaures increase by ± 0.5°C from 38.3-38.6°C, approximately 6.9%-12.8% of the conception rate is lost (Hansen, 2015). This, in turn, produces low conception rates, longer calving intervals and economic losses for the producer (Kornmatitsuk et al., 2008). If the heat index 10 days before estrus is too severe the animal will be affected by a lowered fertility as follicles are directly compromised by the amount of hormone secretions of gonadotropins (Hansen, 2009) disturbing the development of the maturing oocyte (Edwards et al., 2009). Heat stress causes an increase in FSH, which in turn increases the number of small and medium follicles maturing, yet doesn't allow the rise of estradiol, aromatase activity and LH receptor levels in follicles causing delays in ovulation (Rensis and Scaramuzzi, 2003; Roth, 2008; Hansen, 2009). In the event that fertilization were to occur, the embryonic formation would take place at a slower rate or cause embryonic death from abnormal development (Edwards et al., 2009; Hansen, 2009). This is due to an increase in intrauterine hyperthermic stress which would decrease blood flow causing elevated temperatures to the uterus (Rensis and Scaramuzzi, 2003). Because of this, cells in this proximity are designed to destroy those that are malignant or malformed and hyperthermia experienced by the mother the first day of embryo development will most likely cause apoptosis. Therefore, the embryo will not reach blastocyst on day 8 (Hansen, 2007; 2009) and implant into the uterine wall (Rensis and Scaramuzzi, 2003). However, if this were to occur day 3, 5 or 7 of embryonic development the embryo would not be affected because although hyperthermia would be affecting the uterus, heat-shock proteins (HSP) will be present

and only the first cells affected by maternal heat stress would be destroyed (Roth, 2008; Hansen, 2009).

### 3.4.2 Effects of heat stress on sperm

Heat stress not only affects embryonic development but also the development of gametes. Sperm are also affected by the increase in heat specifically during the spermiogenic and meiotic stages of spermatogenesis (Rahman et al., 2011) affecting their quantity and quality (Hansen et al., 2001). During spermatogenesis heat stress affects the thermoregulation of the testicles (Rahman et al., 2011) particularly *Bos taurus taurus* (BT) breeds, such as the Holstein-Friesian. BT breeds are found to have larger amounts of abnormalities in sperm morphology than *Bos taurus indicus* (BI) bulls because they possess scrotal insulation which in turn causes a decrease semen quality (Ashrafzadeh et al., 2013). BI breeds have a better regulation of their testicular vascular cone and morphology (Ashrafzadeh et al., 2013). All parts of the sperm are affected and those which are compromised present a decrease in motility as a result of irregular flagella development (Ashrafzadeh et al., 2013). Meanwhile, those with abnormal heads show an increase in DNA fragmentation (Rahman et al., 2011). Most spermatozoa present pyriform irregularities which also has an effect on acrosomal distribution and interaction (Shojaei Saadi et al., 2013). Yet spermatozoa that are found in the epididymis tend to be greatly unaffected by heat stress (Rahman et al., 2011). Although heat stress affects the development of sperm, the meiosis stage when this process is affected is still not well established (Rahman et al., 2011).

### 3.4.3 Heat dissipation of *Bos taurus taurus* vs *Bos taurus indicus*

*Bos taurus indicus* breeds (BI) such as Zebu cattle are found to have less problems due to heat stress (Hansen et al., 2001; Hansen, 2004; Ashrafzadeh et al., 2013) mainly due to their ability to thermoregulate their bodies more efficiently (Brito et al., 2003; Ashrafzadeh et al., 2013). BI cattle are better adapted to hyperthermal environments because they have larger skin surface area compared to body mass along with a greater amount of sweat glands that are greater in size than those of Holstein cattle (Brito et al., 2003; Hansen, 2004; Ashrafzadeh et al., 2013). Zebu cattle also have sleek and shiny hair that helps in the conduction and convection of heat dissipation (Hansen et al., 2001; Brito et al., 2003; Hansen, 2004; Ashrafzadeh et al., 2013). Further more, BI breed males also have better testicular thermoregulation as was previously mentioned (Ashrafzadeh et al., 2013). It is common practice for breeds to be crossed [*Bos taurus taurus x Bos taurus indicus*] to produce a progeny with better thermoregulation traits in tropical climates although these result in undesirable reductions in milk yields (Hansen, 2004).

### 3.4.4 Effects of heat stress on ovum viability

Heat stress affects ovulation and also affects the oocyte viability although it is not completely clear as to how these are damaged due to the heat exposure (Roth, 2008; Torres-Júnior et al., 2008). The morphology of the Cumulus Oocyte Complex (COC) has an effect on the health and future development of the oocyte (Dey et al., 2012). Exposure of these COC's to hyperthermic conditions during maturation causes the COC

to disintegrate and in turn reduces nuclear maturation leading to apoptosis of the oocyte (Torres-Júnior et al., 2008; Edwards et al., 2009). Thus, the presence of a healthy, intact COC is necessary to complete embryonic development (Dey et al., 2012). There is also a correlation in the duration of the exposure of the COC to elevated temperatures (Edwards et al., 2005). The longer the oocytes are submitted to temperatures 41°C or higher, the less probable it is for these to have an efficient maturation because of the effects on the COC complex (Edwards et al., 2005). Oocytes who are denuded are found to have detrimental effects on the maturation and future fertilization of the oocyte (Dey et al., 2012). The presence of COCs also help in the guidance of spermatozoa to the oocyte and the capacitation of these (Dey et al., 2012). Although BI genotypes are more resistant to hyperthermal stress, these breeds' reproduction is also affected by an increase in temperature and humidity because of the effects on the viability of the oocytes (Torres-Júnior et al., 2008).

### 3.5 Benefits of In Vitro Production of Embryos

Over the past decades *in vitro* production of embryos has increased (Sagirkaya et al., 2006; Salhab et al., 2011) and even though the development capability of transferable embryos is lower than those developed *in vivo*, (Warzych et al., 2007; Salhab et al., 2011) it provides a potential tool to improve reproductive physiology (Keskintepe and Brackett, 1996) and efficiency of cattle (Sagirkaya et al., 2006). Since one of the most detrimental factors for reproductive efficiency is heat stress (Edwards et al., 2005), the *in vitro* production of embryos could by-pass the effects of maternal

hyperthermia on the oocyte which would in turn improve the possibility of conception and development (Rensis and Scaramuzzi, 2003). Although the maturation of *in vivo* oocytes vs *in vitro* oocytes does not show differences in nuclear maturation or fertility (Ali et al., 2004), the retrieval of the *in vitro* oocytes is different and causes a spontaneous restart of the first meiotic arrest (Salhab et al., 2011). These oocytes are generally extracted from follicles that are 2-6 mm which are still 4 to 10 days away from natural ovulation and have not yet attained the developmental capabilities they need to become blastocysts (Ali et al., 2004). However, with the proper handling of the ovaries (Ali et al., 2004) and the improvement of *in vitro* maturation (IVM), embryo production would increase (Salhab et al., 2011).

Therefore, providing hyperthermic-stressed oocytes with the proper *in vitro* maturation culture medium could help improve reproductive efficiency of cattle in Puerto Rico by offering alternative reproduction strategy for optimizing fertility in tropical conditions.

## 4 Materials and Methods

### 4.1 Abattoirs

During this experiment two abattoirs were utilized. Mataderos Hermanos Alvarado, located on the north side of Puerto Rico on Road 10 Km 80.1, Bo. La Planta in Arecibo, is known for slaughtering mostly dairy cattle due to its location in the heart of the Puerto Rico's dairy industry. On the contrary, Ganaderia Santiago is located on the south side of Puerto Rico, on Road 121 Km 12.1, Bo. Susúa in Yauco, and was predominatly used to retrieve ovaries from beef type crossbreds due to the greater number of beef production farms in the area. These abattoirs were utilized over the course of two years during all four seasons [S1: Dec.-Feb. (Winter), S2: Mar.-May (Spring), S3: Jun,-Aug. (Summer) and S4: Sep.-Nov. (Autumn)]. During these seasons the average temperatures in Puerto Rico fluctuated from 20°C - 29°C during S1, 20°C - 30°C during S2, 22°C - 32°C during S3 and 22°C - 31°C during S4 (National Weather Service Forcast Office, 2015). Eighty-seven dairy cattle ovaries (Hostein) and 120 ovaries of beef type crossbreds providing a total of 804 cumulus oocytes complexes (389 dairy and 415 beef oocytes) were collected to be matured *in vitro* (Table 1 and Figure 1).

| Type | S1: Winter | S2: Spring | S3: Summer | E4: Autumn | Total |
|---|---|---|---|---|---|
| Dairy | 58 | 162 | 100 | 69 | 389 |
| Beef | 105 | 70 | 165 | 75 | 415 |
| Total | 163 | 232 | 265 | 144 | 804 |

**Table1: Distribution of cumuls oocyte complex per season and type of cattle**

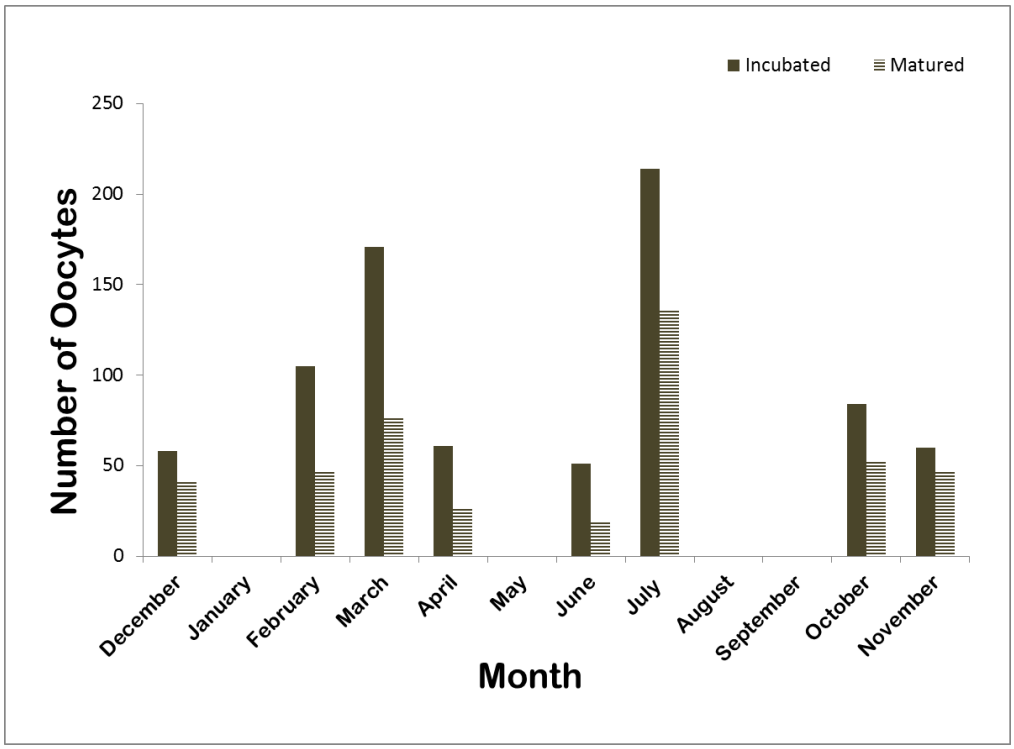



**Figure 1: IVM of oocytes during the months of the year.**

*4.2* Preparations before Processing

Previous to the arrival of the ovary samples to the laboratory, Medium 199 with Hank's salts (500ml; Sigma) was placed in a 37°C water bath along with the L-Glutamine [L-Gln] (1%; Sigma), Penicillin/Streptomycin [Pen/Strep] (1%; Life Technologies) and Fetal Bovine Serum + Heparin [FBS] (2%; Sigma), supplements needed to produce the Oocyte Collection Medium + supplements [OCM+]. While the reactants were warming to its desired temperature of 37°C, the work bench was disinfected by cleaning off all organic material with double distilled water followed by ethanol at a 70% dilution. Once the table was properly cleaned, absorbent paper was placed to avoid the interference of solutions spills during the slashing portion of the oocyte collection procedure.

After properly heated to the required temperature, the supplements were then added to the Medium 199 with Hank's salts under a laminar flow hood to keep the OCM+ as sterile as possible. This solution was then placed over a hot plate along with the beakers used in the collection procedure in order to maintain them at 37°C to prevent temperature fluctuations during the oocyte collection. Scalpel blades (No. 11 and No. 21; Fisher Scientific) and previously autoclaved surgical packs (two blade handles and one hemostat per pack) were also place on the absorbant paper with two beakers (one to hold ovaries in 0.9% saline and another for oocyte collection during slashing).

Approximately two hours before the arrival of the ovaries from the abbatoirs and during the heating of OCM+ reactants, the three Oocyte Maturation Medium treatments (OMM+; OMM1A+, OMM1B+ and OMM2) were prepared and set to equilibrate. The laminar flow hood (Misonix Aura 550) utilized was first cleaned thoroughly by removing all organic material with double distilled water (dd$H_2O$) followed by disinfecting the area with ethanol 70%. Once the area was properly sterilized the reactants and aliquots [Tissue Culture Medium 199 with Earls Salts (OMM-; Gibco), Sodium Pyruvate (Sigma), FBS (Sigma), Pen/Strep (Life Technologies), L-Gln (Sigma), Estradiol (Sigma) and Pluset (FSH and LH at a concentration of one to one; AgTech)] were removed from the refrigerator or freezer, disinfected with ethanol 70% and placed in the sterilized laminar flow hood along with serological pipettes, pipette bulbs, micropipettes, 15ml conical tubes, four well plates (AgTech) and tip boxes. While inside the laminar flow hood the conical tubes and the cover of the four well plates were labeled with their respective OMM+ [A, B or C]. Each treatment prepared consisted of the same base of Tissue

Culture Medium 199 with Earls Salts, FBS (10%), sodium pyruvate (1%), L-Gln (1%) and Pen/Strep (1%) (Moore et. al., 2007a; 2007b). To these the following supplements were added:

1. Medium A, OMM1A+ — Pluset (5µg/µl), Estradiol (25µM).
2. Medium B, OMM1B+ — Pluset ( 25µg/µl), Estradiol (25µM)
3. Medium C, OMM2A+ — Pluset (5µg/µl)

Once each of these was prepared, the conical tubes were inverted various times permitting the reactants to mix. Then a four-well plate was filled with 400µl of each OMM+ treatment in individual wells leaving one empty. Subsequently, the plate and the loose capped conical tubes were placed in a 5% $CO_2$ humidified incubator (SANYO Electric Co., Ltd.; Model MCO-19AIC UV) at 37.5°C for equilibration.

## 4.3 Collection, transportation and preparation of ovaries

Ovaries collected at abattoirs were surgically removed from the rest of the female reproductive tract with a freshly sharpened knife leaving a small amount of extra tissue at the base for handling purposes in the laboratory. As soon as the ovaries were removed they were placed in a clean thermos with a 0.9% saline solution and Pen/Strep, previously heated to 37°C, to ensure the preservation and quality of the ovaries.

Upon arrival at the laboratory, the ovaries were washed with 0.9% saline solution at 37°C, to remove excess blood and/or contaminants that may be transferred to the

collecting medium during the slashing technique. Each ovary was processed by measuring its length and width with a calliper that specified the size in cm. In addition, the surface of each one was thoroughly examined to quantify antral follicles, corpora hemoragicum, corpora lutea and corpora albicans. Once the collection of physical data was retrieved, each follicle was opened using a slashing technique which will be described later. Time from slaughter to oocyte collection was essential due to the fact that the quality or viability of the oocytes diminishes after slaughter (Gordon, 2003; Abdel-Khalek et al., 2010).

## 4.4 Oocyte Collection

Approximately 150 mL of OCM+ were added to a 500 mL beaker and placed on the working bench. A homeostatic forcep was placed at the base of the ovaries for gripping and removal of the excess ovarian tissue with a No. 21 scalpel in order to reduce the amount of tissue that could fall to the medium as this would cause coagulation of the solution (Figure 2). Follicules were then visually assessed for size. Those follicles measuring more than 8-10 mm were opened with a No. 11 scalpel and emptied onto absorbent paper as these also cause coagulation of the medium. The ovaries were then placed over the beaker where follicles 2-8 mm visible on the surface were opened to release the oocyte (Figure 2). Then in order to extract the oocytes, the ovaries were shaken vigorously in OCM+ releasing the oocytes from the ovary to the solution (Figure 2). After processing the ovaries in the same beaker (dairy or beef), more OCM+ was added to the beaker until reaching a volume of 250-300 mL. The

beaker was covered and set to stand for 5 minutes on foam fixtures to allow the oocytes to descend without fluctuating the medium's temperature.

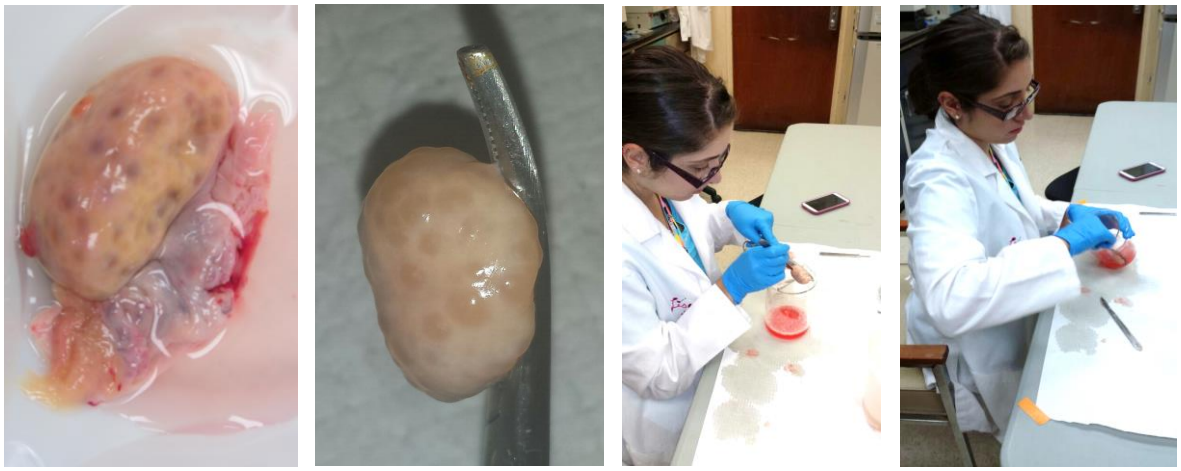



**Figure 2: Slashing and Oocyte Extraction** (A) Ovary after removal from reproductive tract; before slashing. (B) Ovary after tissue removal. (C) Slashing of ovary. (D) Extraction of oocytes from ovary into OCM+.

Meanwhile, the table was prepared for the collection of the oocytes. The absorbent paper and ovaries were disposed, and the working table was disinfected with water and 70% ethanol. Grid search plates (2 plates; Fisher Scientific) and X-plates (2 plates; Fisher Scientific) were placed on the table with other foam fixtures. Again, these were used to avoid the fluctuation of temperature. A 5µl Drummond microdispenser (Thomas Scientific) was desinfected with 70% Ethanol and a sterile glass bore (Fisher Scientific) was placed on the stainless steel wire plunge.

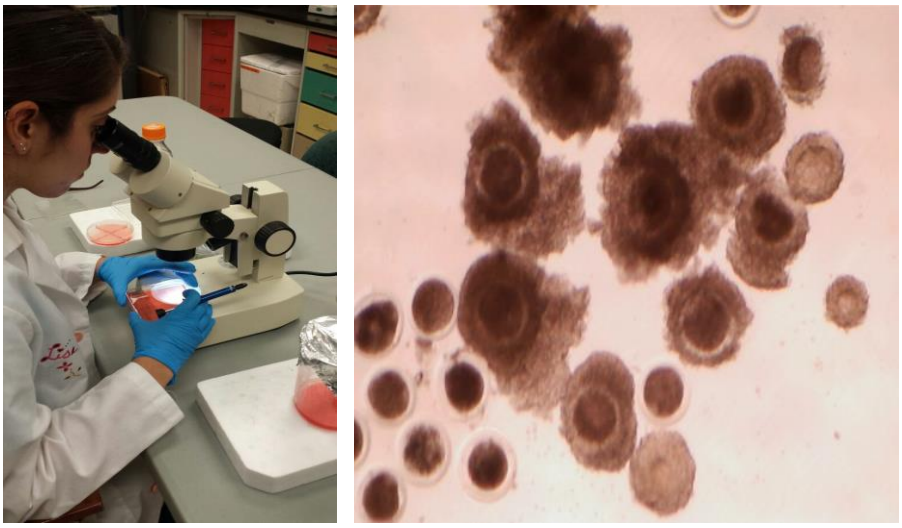
After five minutes, the supernatant was aspirated until reaching approximately 100 mL of OCM+ with the descended oocyte to remove excess debris and secretions from slashing, permitting the oocytes to be in medium (Figure 3) This was done by connecting a Pasteur pipette to a hose connected to a vacuum pump. Once the pump

was turned on, the supernatant was carefully extracted from the top of the medium in a circular motion around the rim to avoid the aspiration of the oocytes. After the aspiration was completed, the volume of the medium was again increased to about 250 mL, allowing it to stand for 5 minutes. The supernatant was removed once more leaving 50-100 mL of the OCM+ solution containing oocytes.

**Figure 3: Aspiration of supernatant**

The OCM+ with the oocytes were then poured into a grid search plate. Observation of the solution with the oocytes was done using a stereomicroscope (AmScope SE305R-PZ). Selection of viable oocytes was performed according to the following criteria: presence of at least one complete layer of cumulus cells without over expansion and a uniform ooplasm. As selected oocytes were collected, they were transferred to an x-plate with OCM+ in each quadrant via a 5µl Drummond microdispenser (Figure 4). After the collection of viable oocytes was completed, they were washed by transferring the oocytes from the first quadrant of the plate to another

leaving behind the debris and non-viable oocytes that may have been suctioned during the selection of oocytes from the grid search plates. These were arranged randomly in groups of no more than 40-50 oocytes. Although many oocytes did not meet the required criteria for maturation, all dairy cattle oocytes were countablized for comparison purposes (Figure 5).



A B

**Figure 4: Oocyte collection for maturation** (A) Collecting COC's from grided search plate to x-plate (B) Collection of oocytes. Denuded, semi denuded, complete COC oocytes

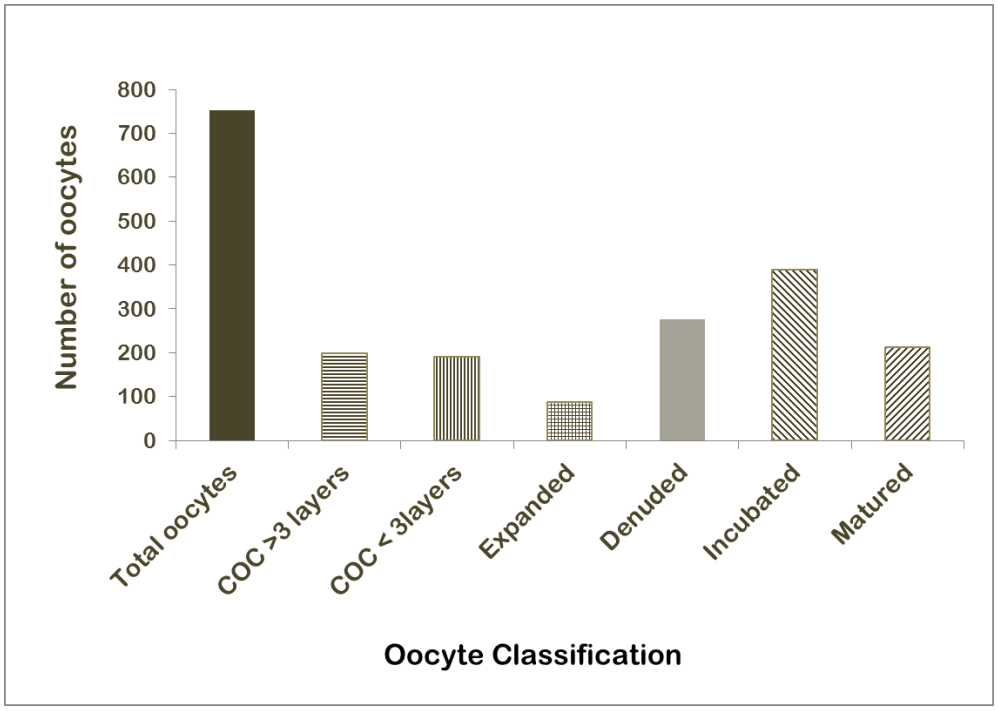



**Figure 5: Classification of dairy cattle oocytes**

4.5 *In Vitro* Maturation (IVM)

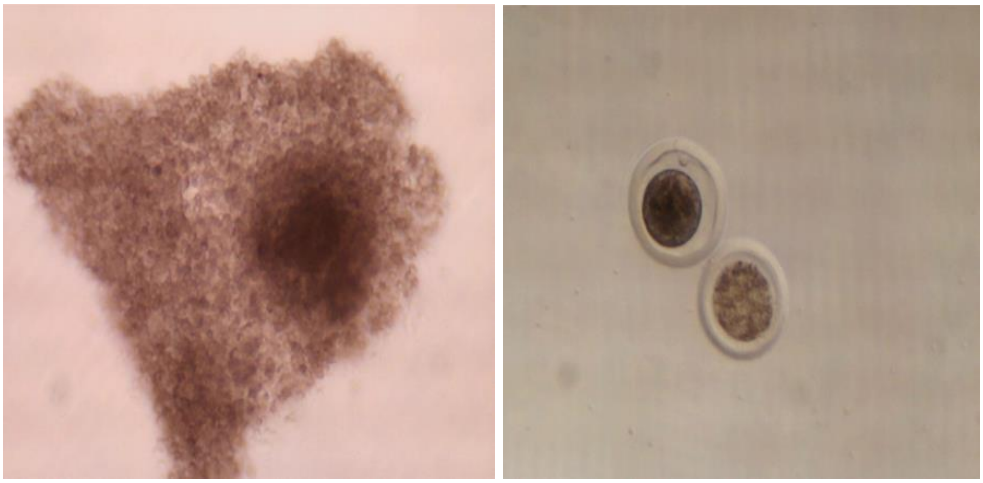
As the groups were made, the four well plates previously prepared with the three OMM+ were removed from the incubator. The oocytes were placed in each well as equally as possible followed by labeling the cover with the amount of oocytes per well (Table 2). The plates were one again placed in the humidified $CO_2$ incubator for a period of 22 hrs.

| Type | Medium A | Medium B | Medium C | Total |
|---|---|---|---|---|
| Dairy | 103 | 126 | 160 | 389 |
| Beef | 142 | 142 | 131 | 415 |
| Total | 245 | 268 | 291 | 804 |

**Table 2: Distribution of cumuls oocyte complex per medium and type of cattle**

4.6 IVM Check

Upon completion of the 22 hour period the oocytes were removed from the incubator. First, expansion of the cumulus cells was visually observed as an indication of possible maturation of the oocytes (Figure 6). Then the oocytes were transferred with a Drummond microdispenser from the OMM+ into labeled conical tubes each containing 1ml of Hepes buffer and 10µl Hyaluronidase. They were placed on a vortex for 5 minutes to induce the removal of the cumulus cells. After 5 minutes, the solution was poured into small petri dishes (3 dishes; Fisher Scientific) repeating the procedure at least two more times adding ± 2 mL of buffer after each inversion to flush the tube and thus ensuring that all oocytes were present for verification. The now nude mature oocytes were observed under a stereomicroscope and a glass utensil made in the laboratory was used in aiding the rotation of the oocytes to ensure the presence or absence of polar bodies, consequently confirming nuclear maturation (Figure 6). The percentage of maturation was then calculated for each medium to be used in data comparison.



A B

**Figure 6: IVM Check** (A) Expanded ovum after 22 hours of maturation. (B) Oocytes stripped of cumulus cells after 22 hours in an incubator. One matured with polar body and another not matured.

## 4.7 Statistical Analysis

The statistical analysis performed in the experiment was done by utilizing SAS (SAS software, Version 9.3, SAS Institute Inc., Cary, NC, USA) in which data was subjected to ANOVA using a General Linear Model. Culture mediums (A, B or C) treatments were chosen in a complete randomized fashion. A total number of 50 (n=50) observations were used and of which the number of oocytes placed per medium were not equal amongst observations. A total of 804 units (oocytes) were observed. The type of cattle (*Bos taurus taurus* or *Bos taurus indicus*) and season of the collection was also noted.

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha_i\beta_j + \alpha_i\gamma_k + \beta_j\gamma_k + \alpha_i\beta_j\gamma_k + \varepsilon_{ijk}$$, where:

$\mu$ = mean of the population

$\alpha$ = maturation media

$\beta$ = type of cattle

$\gamma$ = season

$\varepsilon$ = standard error

Double and triple interactions were removed due to absence of significant differences to allow strength in the degrees of freedom of those who did present significant differences at a $P<0.05$.

## 5 Results and Discussion

Heat stress affects oocyte maturity by lowering the amount of visible polar bodies and increasing apoptosis (Edwards et al., 2005, 2009; Roth, 2004, 2008). In this study, three maturation media with similar components were tested to determine which is best suited for *in vitro* matured oocytes of cattle in Puerto Rico, a sub-tropical environment with a chronic state of heat stress (Table 3). Seasons, cattle type and the interaction of these were also evaluated to determine what effect these variables would have on the *in vitro* maturation of bovine oocytes (Table 3).

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Season | 3 | 0.0837 | 0.0279 | 1.19 | 0.3261 |
| Type | 1 | 0.0033 | 0.0033 | 0.14 | 0.7085 |
| Medium | 2 | 0.2564 | 0.1282 | 5.46 | 0.0080 |
| Season *Type | 3 | 0.3222 | 0.1074 | 4.58 | 0.0076 |

**Table 3: ANOVA** Significant differences only found in the variables Medium and Season x Type

Of the three modified culture medias (TCM199) utilized, Medium A with low concentrations of gonadotropins (FSH and LH; Pluset 5µg/µl) and presence of E2 (25mM) demonstrated to be significantly different than mediums B and C. It showed to have the best maturation rates of oocytes from ovaries of cattle that had been exposed to high temperatures throughout the year presenting a 73% maturation rate of oocyte; meanwhile, mediums B and C demonstrate to have an average of 59% maturity [$P<0.05$; Figure 7]. FSH and LH are frequently added to supplement maturation culture

media because these give root to cumulus cell expansion as well as nuclear and cytoplasmic maturation (Sirard et al., 2007; Wrenzycki and Stinshoff, 2013). E2 is also frequently added to maturation protocols. Although its mechanism of action is not well defined, E2 demonstrates positive effects during IVM (Beker-van Woudenberg et al., 2004).

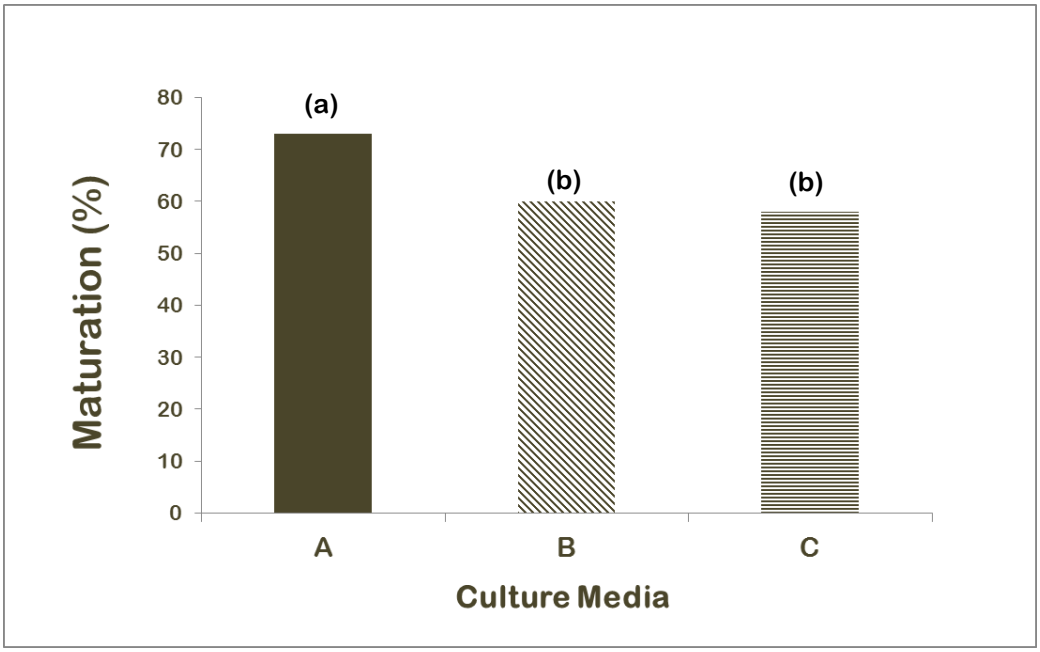



**Figure 7: Effect of the culture medium on *in vitro* oocyte maturation.** Different letters indicate significant differences (P <0.05).

*In vivo* oocytes normally reach nuclear maturation in ovulatory follicles (Lattanzi, 2003) when a LH surge induces the resumption of prophase I arrest before ovulation (Sirard et al., 1989; Edwards et al., 2005). Yet, in *in vitro* embryo production, oocytes used are extracted from 2-8mm follicles and must be matured (Knijn et al., 2002) externally in media, causing a premature maturation (Gilchrist and Thompson, 2007). The positive or negative success rates of *in vitro* maturation (IVM), is dependent upon

the components in the media provided in order to best resemble *in vivo* follicular conditions (Wrenzycki and Stinshoff, 2013). Maturation media contain components such as hormones, proteins, growth factors and nutrients (Harper and Brackett, 1993), therefore the development of a standard medium for oocyte maturtion is not yet definite (Keskintepe and Brackett, 1996).

As was previously mentioned, the mechanism of FSH action upon oocyte maturation is still not completely clear but has been found that it induces the resumption of meiosis. This is due to its effect on germinal vesicle breakdown (GVBD) on cumulus oocyte complexes (COC) and also acts as an activator for cumulus cell expansion, similar to what occurs in LH surges *in vivo* due to its effect on hyaluronic acid in promoting water retention (Sirard et al., 2007). As far as the presence of LH during IVM, it has been found that it increases cumulus cell expansion in treatments with an effective dose of 50µg (Zuelke and Brackett, 1990). This, in turn, shows a carryover effect in which a greater amount of oocytes transform into blastocytes after fertilization vs those who were not treated with LH during IVM (Zuelke and Brackett, 1990). This demonstrates that cumulus cell expansion responds to both FSH and LH supplementation permitting a better maturation rate of oocytes *in vitro*.

E2 has both positive and negative effects on IVM. As Beker et al. (2002) reported, E2 has an adverse effect on oocyte IVM, however they stated that this could be caused by improper spindle organization causing irregular chromosome division affecting embryo development later on. Beker-van Woudenberg et al. (2004) confirmed this although in other studies it was suggested that E2 inhibits apoptosis in other cells such as endothelial, male germ cells and cardiac myocytes. As their study progressed,

they found that E2 does have detrimental effects but mostly on denuded oocytes affecting the spindle of these depending on the time of exposure (Beker-van Woudenberg et al., 2004). Eventhough these results suggest that E2 has effects on spindle formations causing problems with the GVBD, it was found that there was no effect on the nuclear maturation, embryo development or embryo quality in COCs (Beker-van Woudenberg et al., 2004). This promotes the thought that the cumulus cells provide protection to oocytes when exposed to E2 thus demonstrating that IVM is not affected and apoptosis is inhibited (Beker-van Woudenberg et al., 2004).

As it has been proven each individual hormone supplement aids in the IVM of COCs and the use of these together is now in question. Saeki et al. (1991) experimented with IVM mediums with and without the presence of FSH, LH and E2. Even though the maturation rates did not differ among treatments a cross over effect was seen in fertilization (Saeki et al., 1991). Fertilized oocytes matured with FSH, LH and E2 demonstrate to have a greater cleavage rates and blastocysts development (Saeki et al., 1991). Different concentrations of hormone supplements were not evaluated in the investigation performed by Saeki et al. (1991) but gonadotropins and E2 concentrations used were low [LH (5.0µm/mL), FSH (0.50µm/mL) and E2 (1.00µm/mL)] supporting our results.

Although beef and dairy cattle did not demonstrate significant differences in maturation rates [66.6% vs. 61%, respectively; $P>0.05$] (Figure 8) nor did the maturition rates of oocytes during the seasons [S1: 61%, S2:64%, S3: 62% and S4: 69% $P>0.05$] (Figure 9), we can not infer that there are no differences due to the interaction between cattle type and seasons which is significantly different according to the ANOVA that was

previously seen (Table 3). During S1, S2 and S3 no significant differences can be seen between oocytes from dairy cattle or beef cattle (Figure 10). On the other hand, during S4 it is observed that beef cattle have a better maturation rates of oocytes than those of dairy cattle (80% vs 60%, respectively; $P<0.05$). Eventhough S3 does not show signicant differences between the maturation rates of dairy and beef oocytes there is a significant difference between S3: dairy cattle [50%] and S4: beef cattle [80%] ($P<0.05$; Figure 10). Furthermore, there is a 21% reduction in dairy oocyte maturation from S2 to S3 while beef oocyte maturation increased by 13% during the same seasons demonstrating a clear interaction eventhough the maturation rates are not significantly different (Figure 10). Another 12% increase can be observed in beef oocytes from S3 to S4. This may very well be due to their ability to regulate body temperatures more efficiently in response to heat stress (Hansen, 2004).

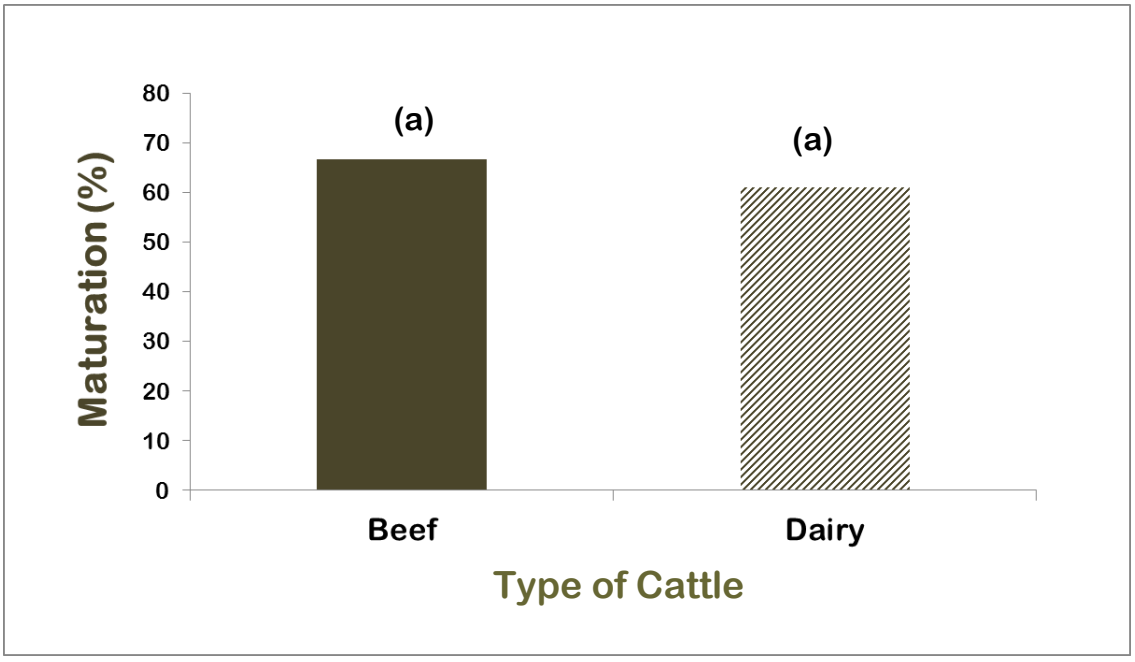



**Figure 8: Differences between types of cattle ($P>0.05$)**

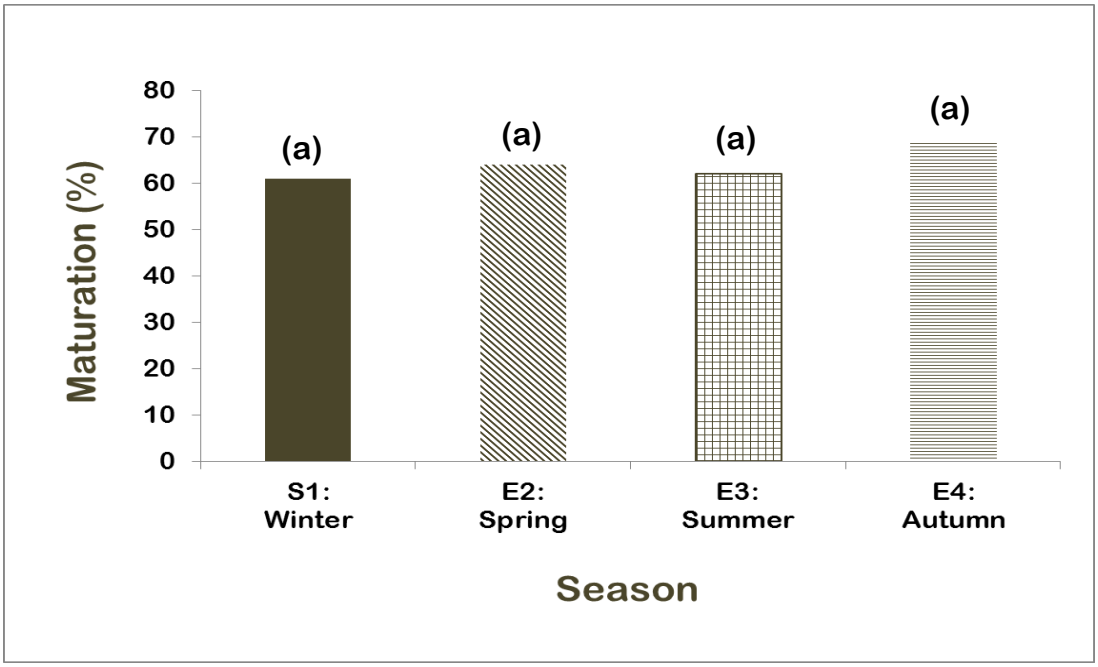



**Figure 9: Differences among seasons (P>0.05)**

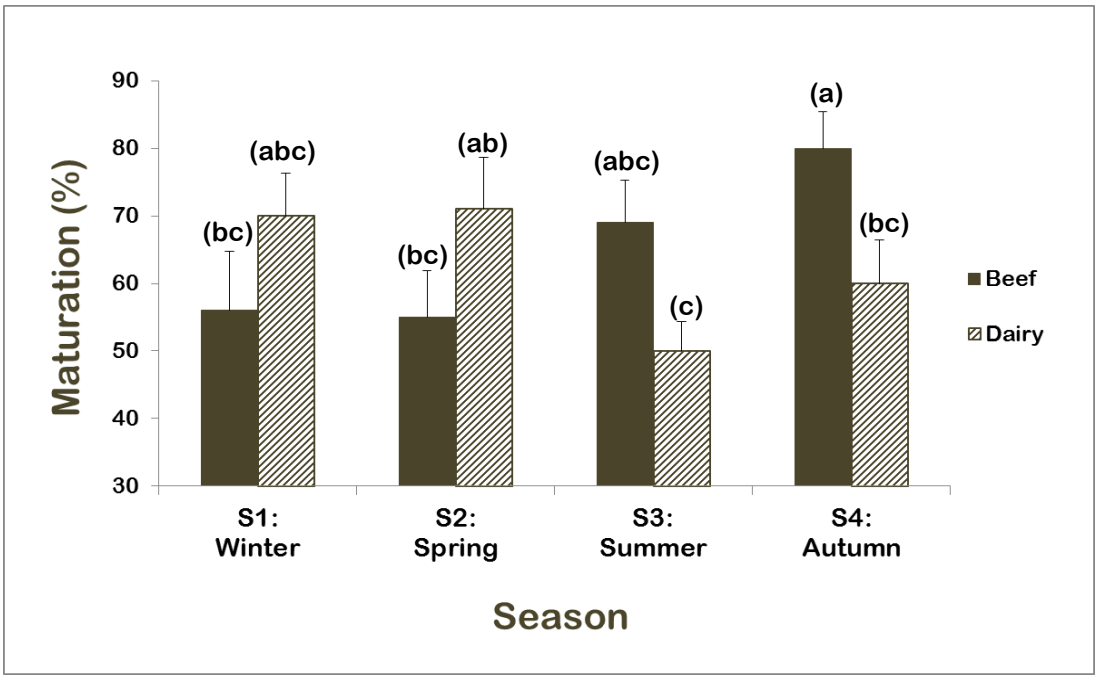



**Figure 10: Effect of seasons on the IVM of bovine oocyte (P<0.05)**

It has been found that at a cellular level, cattle such as *B. taurus indicus* and Senepol have acquired genetic protection to elevated temperatures (Hansen, 2004). Kamwanja et al. (1994) and Paula-Lopes et al. (2003) both found that lymphocytes in beef cattle, such as Brahman and Senepol, are more resistant to thermal stress apoptosis. Embryos of these breeds also demonstrated reduced development and endoooometrial responses when exposed to elevated temperatures (Malayer and Hansen, 1990; Paula-Lopes et al., 2003). Having these genotype responses to thermal stress provides us with the information that proves that beef cattle have genetic mechanisms that help them defend themselves from and environment of chronic heat and humidity. This could be that there is a thermo-tolerant mechanism that is activated during higher periods of heat stress in beef cattle while dairy cattle use their defense year round causing them to have better maturation rates in cooler months vs. beef cattle. This leads to question what exactly is activated in the genome of beef cattle that causes them to mature better in severe heat vs moderate heat.

## 6 Conclusion

These findings suggest that maturation media supplemented with 5µg/µl of gonadotropins and 25µM of estradiol will help the maturity rates of *in vitro* matured oocytes. Oocytes from dairy cattle will improve with these supplements if added to the maturation media during seasons of moderate thermal stress while those of beef cattle are enhanced during severe thermal conditions. These results also provide us with information that cause undefined questions to be answered in future investigations of genomic activation in heat stressed beef type cattle.

## 7 Implications and Recommendations

As the results from this investigation demonstrate, maturation rates are improved by supplementing TCM199 with Pluset in concentrations of 5µg/µl and presence of estradiol. The effect of these gonoadotropins can also be evaluated by adding growth factors such as epidermal growth factors in order to keep optimizing media for better maturation rates of bovine oocytes. Future studies should also include a profounder investigation on what induces beef type cattle defense against thermal stress since they apparently have a mechanism that allows them to tolerate high temperature more efficiently. These future investigations will help improve the reproductive efficiency of cattle in Puerto Rico due to the climatically stress induced conditions of constant heat and humidity and in turn helping the productivity of these animal.

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