Ovulation and corpus luteum development after synchronization with a progesterone delivery device (CIDR) plus prostaglandin or prostaglandin alone under tropical conditions in hair breed sheep

By

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Thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

ANIMAL SCIENCE

UNIVERSITY OF PUERTO RICO

MAYAGÜEZ CAMPUS

2017

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Abstract

This investigation has focused in studying differences in the ovulation rate and the corpus luteum (CL) formation by utilizing two ovulation synchronization protocols based on the use of a controlled internal drug releasing (CIDR) device combined with prostaglandin (PG), or administration of PG without a CIDR. A total of 40 crossbreed Barbados Blackbelly x White Virgin Island ewes were used for this study. Ewes were maintained in a paddock of 100 m^2 at Ross University School of Veterinary Medicine (17.3º N, 62.7º W) in St. Kitts. They were assigned randomly at different stages of the estrous cycle and separated into two groups (20 ewes / group). In the CIDR+PG group, ewes received an application of a CIDR device for 7 days and PG (10 mg, i.m., Lutalyse, dinoprost tromethamine) was administered at CIDR device removal. The PG group received an administration of PG (10 mg, i.m., Lutalyse, dinoprost tromethamine) with 10 days apart. After treatment, ovaries were examined daily, for 21 days, by transrectal B-mode ultrasonography using a 7.5 MHz lineal array transducer to determine ovulation and CL development. Ovulation was defined as the detection of a large follicle (> 4 mm) at one examination, and was absent at the next examination. The interval from treatment to ovulation was considered as the time between CIDR device removal for Group CIDR+PG and the second injection of PG for the PG group. The day-to-day profile of CL area affected by treatment ($p < 0.002$), and by time ($p < 0.0001$). The CL area was greater in single ($p < 0.01$) than in double ovulations observed in the CIDR+PG group. Plasma progesterone concentrations were also affected by treatment ($p < 0.0001$) and by time ($p < 0.0001$). In conclusion, application of CIDR+PG to ewes under tropical conditions resulted in a high degree of synchronized ovulation and a visually enhanced structural development of the CL, as compared to PG alone, especially in those animals with single ovulation.

Resumen

Esta investigación se ha enfocado en estudiar las diferencias en la tasa de ovulación y de la formación de cuerpo lúteo (CL) al utilizar dos protocolos de sincronización de la ovulación basados en el uso de un dispositivo de liberación controlada de fármacos (CIDR) combinado con prostaglandina (PG) o por administración de PG sin un CIDR. Un total de 40 ovejas cruzadas Barbados Blackbelly x White Virgin Island fueron utilizadas para este estudio. Las ovejas fueron mantenidas en un corral de 100 m^2 en la Universidad de Ross Escuela de Medicina Veterinaria (17.3º N, 62.7º W) en St. Kitts. Estas fueron asignadas al azar en diferentes etapas del ciclo estral y separadas en dos grupos (20 ovejas / grupo). En el grupo CIDR+PG, las ovejas recibieron una aplicación de un dispositivo CIDR durante 7 días y se les administró PG (10 mg, i.m., Lutalyse, dinoprost trometamina) al retirarse el dispositivo CIDR. El grupo PG, recibió una administración de PG (10 mg, i.m., Lutalyse, dinoprost trometamina) con 10 días de diferencia. Después del tratamiento, los ovarios se examinaron diariamente, durante 21 días, mediante ultrasonografía transrectal de modo B con un transductor lineal de 7.5 MHz para determinar la ovulación y el desarrollo de CL. La ovulación se definió como la detección de un folículo grande (> 4 mm) en un examen, y que estaba ausente en el próximo examen. El intervalo entre el tratamiento y la ovulación se consideró como el tiempo transcurrido entre la eliminación del dispositivo CIDR para el grupo CIDR+PG y la segunda inyección de PG para el grupo PG. El perfil diario del área CL se vio afectado por el tratamiento ($p < 0.002$) y por el tiempo ($p < 0.0001$). El área del CL fue mayor en las ovulaciones simples ($p < 0.01$) que en las dobles observadas en el grupo CIDR+PG. Las concentraciones plasmáticas de progesterona también fueron afectadas por el tratamiento ($p < 0.0001$) y por el tiempo ($p < 0.0001$). En conclusión, la aplicación de CIDR+PG a ovejas en condiciones tropicales resultó en un alto grado de ovulación sincronizada y un desarrollo estructural visualmente mejorado del CL, en comparación con el grupo PG, especialmente en los animales con ovulaciones simples.

To my whole family which gave me unconditional support along the way, for hearing me and having the patience to do so.

In loving memory of my grandparents; the love for their family and for Mother Nature, inspired me to do this and to love without limits.

"We're still pioneers, we've barely begun. Our greatest accomplishments cannot be behind us, cause our destiny lies above us." -Interstellar

Acknowledgments

I would like to thank the University of Puerto Rico at Mayagüez, for accepting me in the Graduate Program of Animal Science and the Ross University School of Veterinary Medicine for permitting me to complete this work at their facilities.

I would like to thank the graduate committee members, to Dr. Latorre for always being there to support me; for Dr. Sánchez for giving me counseling, and especially, to my research advisor Dr. Arcelay for taking me under your guidance. Thanks to Dr. Ratto for encouraging me to fulfill this research project, and to have the patience to help and guide me.

I want to give special thanks to the great supporting personnel from both universities. Thank you Silvia, Don and Ximena, for making me feel welcomed in St. Kitts. At the UPRM, I want to thank Sonia, Linda, Luisa and others for their help and words of wisdom.

Once again, I would like to thank my lovely family for unconditionally being there.

"¡Antes, Ahora y siempre Colegio!"

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List of Abbreviations

anterior pituitary	AP
Brightness mode	B-mode
Body Condition Score	BCS
Controlled Internal Drug Release	CIDR
corpus luteum	CL
Enzyme-Linked Immunosorbent Assay	ELISA
equine Chorionic Gonadotropin	eCG
estrogen	E_2
Follicle Stimulating Hormone	FSH
grams	g
Gonadotropin Releasing Hormone	GnRH
hour (time)	h
for example	i.e.
intramuscular injection	i.m.
kilogram (weight unit)	kg
Luteinizing Hormone	LH
milligrams	mg
megahertz	MHz
milliliter	mL
millimeter	mm

square millimeter	mm^2
Multiple Ovulation Embryo Transfer	MOET
nanogram per milliliter	ng/mL
number	n
picogram per milliliter	pg/mL
progesterone	P_4
prostaglandin	PG
p-value (probability)	p
Standard Error of the Mean	SEM
Statistical Analysis System	SAS
Superior Cervical Ganglion	SCG
suprachiasmatic nuclei	SCN
Timed Artificial Insemination	TAI

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1. Introduction

The Eastern Caribbean consists of numerous islands that are primarily supported by tourism and some kind of agriculture. Saint Kitts and Nevis, West Indies are one of the Eastern Caribbean island that was primarily sustained by a government controlled and regulated by the production of sugar cane (Momsen & Mill, 2017). In 2005, the government of St. Kitts and Nevis discontinued the commercial production of sugar cane, because it was not sustainable (IICA, 2011). Since the sugar cane industry was the main source of employment and income for the general population of St. Kitts and Nevis, the government launched a Sugar Adaptation Strategy (Stanley, 2010). Under St. Kitts and Nevis Department of Agriculture, this program was developed to create and promote crop and livestock production programs that could generate a more sustainable and diversified agricultural economy (Stanley, 2010).

In these Eastern Caribbean island, small ruminants are an important livestock and serve as a vital source of food and income (Sinn et al., 1999). It has been reported by Stanley (2010) that St. Kitts and Nevis is the net importer of sheep and goat meat products; with a total of 12% in local production; making consumption greater than the availability. Still, there is need to find more effective and efficient ways to increase overall production. To improve production of small ruminants with enhanced meat, milk or wool yield per animal per year, it is necessary to have a fundamental understanding of the basic reproductive physiology of sheep and goats under the environmental conditions in the Caribbean.

The use of estrous synchronization is known throughout the world as a helpful method to enhance breeding efficiency in the small ruminant and other livestock industries (Knights et al., 2011). Small ruminants are seasonal breeders and typically their offspring are born in spring (late January through early February; (Gordon, 1997)). This is seen mostly in temperate regions or in countries found in extreme latitudes like Spain, Australia, and South America (Rosa & Bryant, 2003). In these template regions of extreme latitudes, seasons are marked by extreme changes in photoperiods, presenting shorter days by the fall-winter and longer days by spring-summer. These changes in photoperiod affect the reproductive cycle in small ruminants since they are photoperiod dependent (Abecia et al., 2012). Sheep and goats are short-day breeders (Abecia et al., 2012; Goldman, 1999) meaning that their reproductive activity takes place in the beginning of fall, and should be lambing by the end of winter (Gordon, 1997).

It is crucial to learn how to effectively perform reproduction practices in small ruminants, these are important factors to increase profitability and productivity (Martin et al., 2004), in developing countries. This has been an issue since these animals present an aseasonal breeding pattern under tropical conditions (Rosa & Bryant, 2003). Reproduction management is challenging when livestock exhibit a "throughout-the-year" breeding pattern (Abecia et al., 2012); thus implies that many ewes will be in estrus in different moments of the year. Although this inconvenience is present, these ruminants tend to be very resilient in poor environmental conditions, and do not require labor-extensive work or high capital investments. For that reason, it makes sheep and goats to

serve as a possible vital source of food and income for the local small farms and the economy.

Thus, the main objective of this work is to characterize and evaluate basic aspects the of reproductive physiology and endocrinology of the sheep that inhabit in St. Kitts and Nevis using non-invasive methodology thus applying conventional assisted reproductive techniques to enhance domestic production. This could potentially reduce the import of sheep products and provide a self-sustaining population of these animals that will benefit the locals of the island with jobs, income, and food. It is essential to develop new methods to improve reproductive efficiency and make a profound impact on animal production and their sustainability.

2. Objectives

- To compare the effects of two estrous synchronization protocols, one based on an intravaginal progesterone (CIDR) device combined with PG and another based on two PG administrations, and no CIDR, on the reproductive performance of ewes under tropical conditions.
- Reproductive performances to be included are based on ovulation rate, interval from treatment to ovulation, size of pre-ovulatory follicle, corpus luteum development and plasma progesterone concentration.

3. Literature Review

3.1. Ewe Reproductive Physiology and Seasonality

With the scientific name *Ovis aries,* sheep are categorized as small ruminants that present a seasonal polyestrous estrous cycle in temperate climates (Ali et al., 2006; Bartlewski et al., 2011; Rosa & Bryant, 2003). Female sheep enter puberty approximately at an age of 7 to 8 months, when a 70% of mature body weight is reached (Frandson, 1986). The peculiar estrous cycle of the ewe is stimulated by changes of photoperiod. It has been observed that the photoperiod can modulate growth (Abbot et al., 1984; Schanbacher & Crouse, 1980; Tucker et al., 1984) and the onset of reproductive function (Foster et al., 1985) in these animals. When the ewe is exposed to the shortening of days in autumn will stimulate the initiation of its estrous cycle (Abecia et al., 2012). Ewes can go through 20 estrous cycles in one reproductive season, each with an average length of 16 to 17 days (Contreras-Solis et al., 2008). If copulation occurs, the fetus will develop through winter and be born at the beginning of spring, with an average gestation period of 5 months, where there should be plenty of food available (Gordon, 1997). This will allow the offspring a better chance of survival. Most research on the reproductive physiology in sheep are done in breeds from temperate environmental conditions at relatively high latitudes (>35° N), where animals display a distinct breeding season than in the tropic, thus presenting estrous during the fall and winter (Abecia et al., 2012; Rosa & Bryant, 2003).

Since few studies have evaluated reproductive efficiency in sheep under subtropical (Ali et al., 2009) or tropical conditions (Ali et al., 2006; Contreras-Solis et al., 2008) there is a need for more extensive evaluations of the ovarian dynamics in these mid-latitudes. For example, to our understanding the environmental impact of seasonal changes (i.e. day length, temperature and availability of forage) on estrous cyclicity and ovarian dynamics in sheep and goats on St. Kitts and Nevis is not known. The season in these Caribbean Islands (mid-latitudes; 20° N to 20° S) does not show considerable differences of photoperiod during the year; with only 2 to 3 hours of light differences between each season. At a latitude of 17° N and annual average temperature of approximately 27°C, the small ruminants from these islands may have adapted to the photoperiod and may present estrous throughout the year. This behavior has been observed in ewes at equatorial latitudes such as Ethiopia (9° N) and Mexico (23° N); in natural field environments (Rekik et al., 2016).

Alternatively, abundant or limited availability of forage during the wet or dry seasons may still influence the need for seasonal breeding especially, since a deficiency in nutrition is a main factor associated with prolonged anestrous and anovulatory periods that has resulted in reduced fertility and prolificacy (Delgadillo et al., 1997). The big question here is if the lack of significant changes of photoperiod in the tropics can affect the way that small ruminants respond to exogenous hormonal protocols that have been previously evaluated in temperate regions. Understanding how these protocols function in the tropics, it will help farmers to achieve optimal livestock production of small ruminants in the tropical islands.

3.1.1. Management and Seasonality Effect in Ewes

It has been shown that some breeds of sheep can be highly prolific, while others present limited reproductive performance. Ewes are classified as a prolific breed, have a tendency to give multiple offspring in one lambing during one season (Bartlewski et al., 2011). Tropical breeds that are categorized as prolific are Barbados Blackbelly, White Virgin Island, Javanese Thin-Tail (Sutama et al., 1988), Finnish Landrace, and Romanov sheep (Campbell et at., 1995). Meanwhile, non-prolific breeds give only one offspring per lambing per season including the Australian Merino (Campbell et al., 1995) and Western White Face ewes (Barrett et al., 2002).

In ewes, the typical signs of estrus could be the rapid movement of the tail, standing heat position and clear vulva secretions, meaning that estrogen has reached its peak, making the ewe receptive to the male (Rosenfeld & Heide, 2008). However, some ewes do not present signs of estrus, thus requiring the use of a teaser ram to help detect females in heat (Avdi & Chimeneau,, 1998; Ortman, 2000). Usually males have undergone an epididymectomy or vasectomy since they are only used to detect estrus and not take part of a successful copulation (Godfrey et al., 2001). If the availability of a ram is limited, then the use of multiple techniques could be implemented to determine if an ewe is in heat. This type of management is very important since it is critical to determine successfully if the ewe has responded to a synchronization protocol, this permitting a cost-effective program in the farm.

Categorized as short-day breeders, ewes are stimulated by an increase of melatonin when nights become longer (Abecia et al., 2012). Melatonin concentrations have a considerable influence in controlling its estrous cycle. The available photoperiod in the animal's environment will enter the retina, sending signals to the pineal gland where the suprachiasmatic nuclei (SCN) are found (Rosa & Bryant, 2003). The SCN induce the synthesis of the pineal melatonin and eventually result in what is known as the circadian rhythm in mammals (Yellon, 1992). When photoperiod decreases, few biological signals reach the pineal gland, and in ewes, melatonin levels increase thus allowing estrus to set in (Goldman, 1999). These trigger changes the hypothalamus to increase the production of gonadotropin releasing hormone (GnRH), therefore increasing the levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior pituitary gland (AP), eventually giving rise to the onset of the seasonal estrous cycle (Rosenfeld & Heide, 2008; Senger, 2005). In temperate conditions, this is an expected reproductive system behavior. However, in tropical conditions (with the absence of drastic photoperiod changes) the ewe present estrous cycles year around, thus, making reproduction management difficult for maintaining homogenous gestating female groups in the farm.

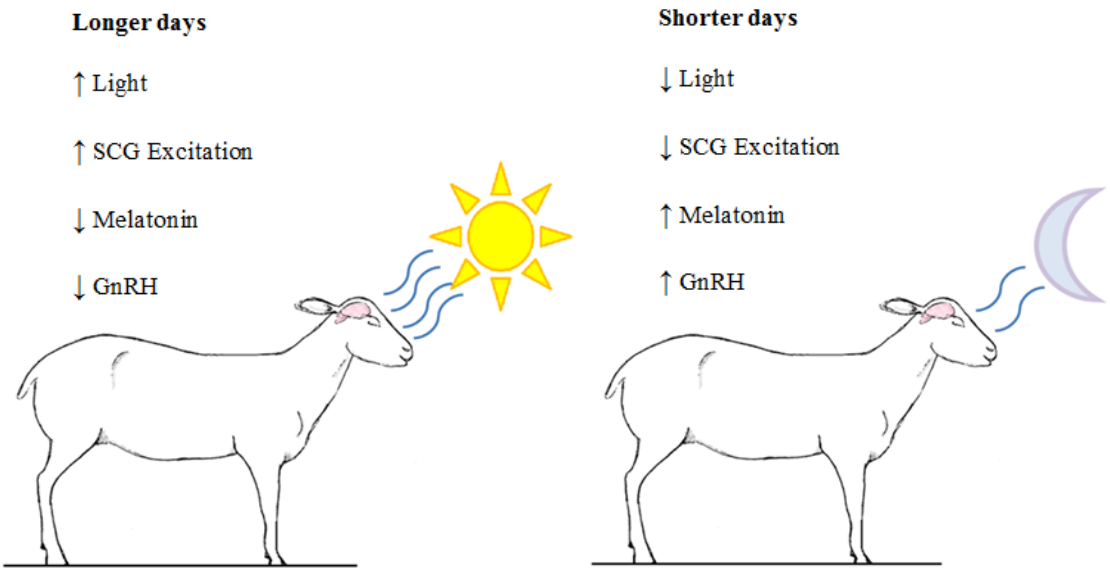

Fig. 1. The estrous cycle response in different photoperiods, in the ewe. Superior Cervical Ganglion (SCG) depends on the excitatory neurons stimulation for it to send signals to the pineal gland. Melatonin levels are influenced by these high or low frequency of the SCG.

3.1.2. *Neuroendocrine Regulation in Ewe Estrous Cycle*

Systemic and local changes triggered by environmental or hormonal factors can control processes such as follicle emergence and growth, ovulation, corpus luteum development and regression, all of which are regulated by the hypothalamic-pituitary-ovarian axis (Contreras-Solis et al., 2009). The estrous cycle is divided in different phases consisting of the proestrus, estrus, metestrus and, diestrus (Goodman, 1994).

3.1.2.1. Follicular Phase

The first phase of an estrous cycle is called the follicular phase, consisting of proestrus and estrus (Senger, 2005). Proestrus consists of the complete regression of the corpus luteum (CL) and an increase of Estrogen (E_2) concentrations (Bartlewski et al.,

2017). This elevation of E_2 happens because FSH is released into the bloodstream from the AP gland (Driancourt, 2001). High concentrations of E_2 will develop the follicles until they reach the tertiary follicle structure (Rosenfeld & Heide, 2008). The estrus phase is when the animal presents sexual receptivity and ovulates (Senger, 2005). The concentrations of E_2 in the bloodstream reach a threshold inducing ovulation, due to the increase of LH secretions (Yellon et al., 1992). Also, the AP responds to these elevated concentrations of E_2, creating an ovulatory surge of LH, eventually culminating in the rupture of the mature follicle (Senger, 2005).

3.1.2.2. Luteal Phase

In the metestrus, the CL begins its development in the ovary, and progesterone (P_4) is secreted into the blood stream, inducing an increase of endometrium vascularity to sustain a possible implantation (Senger, 2005). This P_4 will enter the bloodstream and create a negative feedback to the hypothalamus, this causing a suppression of GnRH (Arroyo-Ledezma et al., 2013). By Day 10 to 14 the CL is fully functional and the elevated P_4 concentrations are secreted, this stage is called the diestrus phase (Slayden et al., 1989). If pregnancy does not take place, prostaglandin $F_{2\alpha}$ (endogenous prostaglandin) is secreted from the endometrium, causing luteolysis (Ginther et al., 2009; Goravanahally et al., 2007). This process is rapid, thereby decreasing progesterone concentrations (Shrestha et al., 2010). This process will enable the hypothalamus to secrete GnRH again, permitting the recruitment and selection of antral follicles that could lead to possible ovulation (Bartlewski et al., 1999a; Rosenfeld & Heide, 2008).

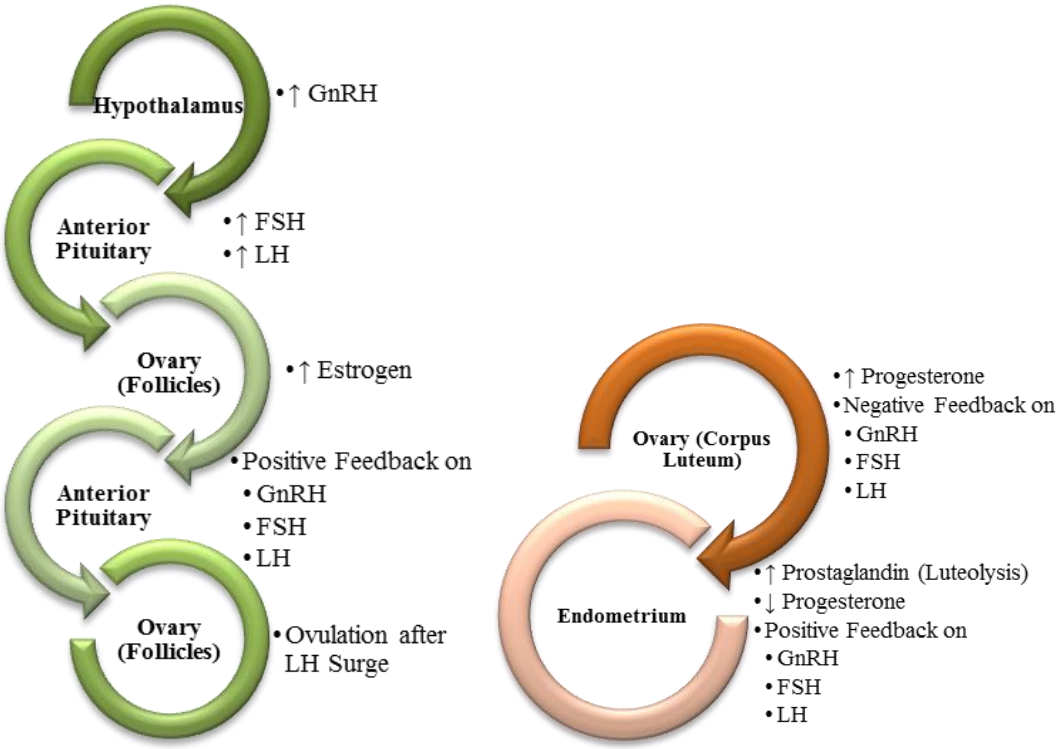

Fig. 2. The Hypothalamic-pituitary-ovarian axis pathway during the estrous cycle of a cycling ewe. Green arrows (left) represent the follicular phase and orange arrows (right), the luteal phase.

3.2. Implication of Estrous Synchronization Protocols use in Small Ruminants

Estrous synchronization is referred as the control of the female neuroendocrine reproductive cycle to induce estrus in the animal within a time frame of 24-96 hours after the protocol's completion (Ali et al, 2009). This occurs through the administration of different types of hormones to the female body thus, affecting its hypothalmic-pituitary-gonadal axis (Contreras-Solis et al., 2009). Protocols for estrous synchronization can bring the majority of livestock to a same stage of estrous cycle in a determined time

(Dudhatra et al., 2012; Knights et al., 2011). This useful management tool for programmed breeding allows programmed feeding easier for management of ewes at the same stage of gestation (Dudhatra et al., 2012). Also, this synchronization brings about desirable shorter period of lambing, permitting higher production efficiency in the farm (Menchaca & Rubianes, 2004). When lambing occurs in a synchronized way, it is convenient for the management in the farm, since the expected date of lambing can be predicted; this also reduces the spreading of births and weaning over a shorter period of time. This being said, a larger group of lambs will be at a homogenous age, facilitating the farmer at the moment of selling.

Some reproductive management tools such as synchronization of estrous in sheep and goats, have been widely used to provide induced reproductive activity (Safdarian et al., 2006), even during periods of seasonal anestrous and lactation (Amorim et al., 2007; GdeNicolo et al., 2007). The methods used for synchronization of estrous and stimulation of follicular development in small ruminants include administration of prostaglandins (PG) (Uribe-Velazquez et al., 2008; Weems et al., 2006), P_4 and equine chorionic gonadotropin (eCG) (Menchaca et al., 2007). These treatments are mostly delivered via intramuscular (i.m.) administration (Holtz et al., 2008; Lopez-Sebastian et al., 2007; Uribe- Velasquez et al., 2008) or via absorption with the use of intravaginal devices (Abecia et al., 2012; Jackson et al., 2014; Oliveira et al., 2016). In some instances, the "ram effect" could help to stimulate the group of ewes into heat. Recent studies show that this method causes a positive effect on the ewes in tropical conditions, which are normally in an out of the breeding season phase (Wheaton et al., 1992).

To verify how the animal is reacting to the protocol administered, a transrectal ultrasonography is used. This method is well established and documented to evaluate the efficiency of protocols of estrous synchronization (Bartlewski et al., 1999b; Duggavathi et al., 2003; Evans, 2003; Evans et al., 2000; Ginther et al., 1995; Seekallu et al., 2010). Correspondingly, transrectal ultrasonography is used to study the morphological development of follicles and corpus luteum in sheep (Bartlewski et al., 1999b; 2000; Duggavathi et al., 2003). This last technique provides a basic understanding of the structural and functional relationships associated with ovarian dynamics during the estrous cycle when induced by a synchronization protocol.

3.2.1 Types of Exogenous Hormone Treatments in Ewes

Synchronization of estrous in sheep and goats depends on factors such as controlling the luteal phase or the rapidly developing follicles during the follicular phase (Ali, 2007; González-Añover et al., 2007; Menchaca et al., 2007). Estrus synchronization protocols are used to mimic the physiological process that could occur during the natural breeding environment of the animal. As mentioned before, in ovine reproduction, some of the most commonly used hormonal treatments use intravaginal devices that are coated with P_4 (Abecia et al., 2012) or administer exogenous hormones like GnRH, eCG and PG via i.m. injection. Many of these synchronization protocols decrease P_4 plasma concentrations to less than 1 ng mL^{-1} before the treatment has ended, eventually leading to ovulation (Uribe-Velásquez et al., 2008).

The use of GnRH, for estrous synchronization, helps to accelerate the follicular growth by enhancing LH pulse frequency (Rubianes et al. 1997). The administration of eCG hormone will induce ovulation for small ruminants (Menchaca et al., 2007). However, after multiple uses of exogenous eCG to the animal, this hormone can cause side effects, thus creating ovarian follicular cysts (Viñoles et al., 2001). Because of the resulting low pregnancy rates, the use of eCG has declined for purposes of estrous synchronization (Olivera-Muzante et al., 2011).

3.2.1.1 CIDR+PG Protocol

A Controlled Internal Drug Releasing (CIDR) device is an intravaginal device, used in sheep, made out of silicone, which is coated with 0.3 g of progesterone (Abecia et al., 2012; Jackson et al., 2014; Oliveira et al., 2016). This type of estrous synchronization protocol has been approved in the United States since 2009 (FDA, 2009). There are a variety of protocols in which this CIDR device is used; mostly these are called short-term or long-term protocols. The CIDR devices can be used for long-day treatment protocols, of 12-14 days (Ainsworth & Downey, 1986; Wheaton et al., 1993) and in short-day treatments of 6-10 days (Arroyo-Ledezma et al., 2013; Oliveira et al., 2014). It has been demonstrated that CIDR devices have a mayor contribution in hair sheep under tropical conditions, when left for a time lapse of 6 days (Arroyo-Ledezma et al., 2013). According to Arroyo-Ledezma (2013) investigation, for an 11-day protocol, a marked decline of P_4 concentration by Day 6 with the CIDR device inserted, suggests that it is not feasible to maintain it for a longer period of time since the body will not process the

additional P_4. This shows that the short protocol with the CIDR device coated with P_4 is a preferred type of estrus synchronization.

When P_4 from the CIDR device is absorbed by the animal, it triggers an important endocrine signal that governs the antral follicular development and ovulation in sheep (Bartlewski et al., 2017). The P_4 concentrations should decrease to 0.5 ng/mL after removal of the CIDR device (Gok et al., 2016). These types of hormonal treatments will induce a decrease of LH surge, since at the beginning of the treatment there is high concentrations of P_4 (Goodman & Karsh, 1980) causing a possible follicular regression. Also, the time of administration of the hormonal treatment can affect the follicular wave development of the next estrous cycle. When the CIDR device is removed, a rapid decrease of P_4 concentration takes place, thus simulating the regression of the current CL, and promoting ovulation within hours of the device removal.

3.2.1.2 PG Protocol

There are various types of commercial PG are hormones that have a potent luteolytic effect in ruminants, causing the regression of the CL, eventually inducing estrus in the animal (Light, 1994). With the administration of the first PG i.m. injection, a lysis effect is observed on the CL occurs between the first 5 days. Until approximately Day 11, in the estrous cycle, the functional CL will then start to diminish in size due to the second PG administration, also meaning that the P_4 concentrations will decrease as well (Arroyo-Ledezma et al., 2013). It is possible that ovulation could occur if a mature CL is present when the first i.m. PG injection is administered. It is also possible for the animal to enter estrus before the second injection, a regression of the corpus luteum

followed (Rekik et al., 2016). It is important that PG injections be administered in an interval of 9-10 days, so that most of the animals be in the midluteal phase by the second injection; permitting the animal to ovulate (Contreras-Solis et al., 2009).

The use of PG analogue hormones is convenient since it can be used year around, which is crucial for tropical sheep breeds since they do not present seasonal anestrous (Navarro et al., 1985). The use of PG is known as an alternative for estrous synchronization, having the advantage of a short life span in the body after administration. This ensures that no residue will be left in the body, since the PG metabolizes completely in the lungs after a short period of time (Davis et al., 1980; Kindahl, 1980; Light et al., 1994; Piper et al., 1970). This makes the use of PG a desirable protocol when it comes to selling the animals for meat, since there is little to no chance to find residue of chemicals in the body.

The mechanism of the CIDR+PG protocol is to inhibit the secretion of GnRH, with this the secretion of LH. This will prevent the proper development of the antral follicles and no ovulation can occur with the presence of the CIDR. Ovulation can only occur with the removal of the CIDR, eliminating the inhibition of GnRH (Dogan et al., 2004). The PG protocol's aim is to induce a regression of the CL, permitting the follicular wave to develop at a more rapid pace (Arroyo-Ledezma et al., 2013). However, estrus detection in PG protocols has an interval of 4-5 days, rendering this protocol for timed artificial insemination (TAI) would be unfitting (Olivera-Muzante et al., 2011). This can be encompassed within a Multiple Ovulation Embryo Transfer (MOET)

regimen for sheep and goats (Menchaca & Rubianes, 2004) to effectively enhance animal production.

The implementation of these short protocols in hair sheep at equatorial latitudes have shown to be a promising methods of management (Arroyo-Ledezma et al., 2013) but, to our knowledge, their efficiency have never been confirmed in tropical islands. Moreover, an outreach to the sheep and goat producers in St. Kitts and Nevis Department of Agriculture, can keep people informed of the practical applications of these studies; improving current management practices and increasing meat, milk or wool yield per animal per year in the future.

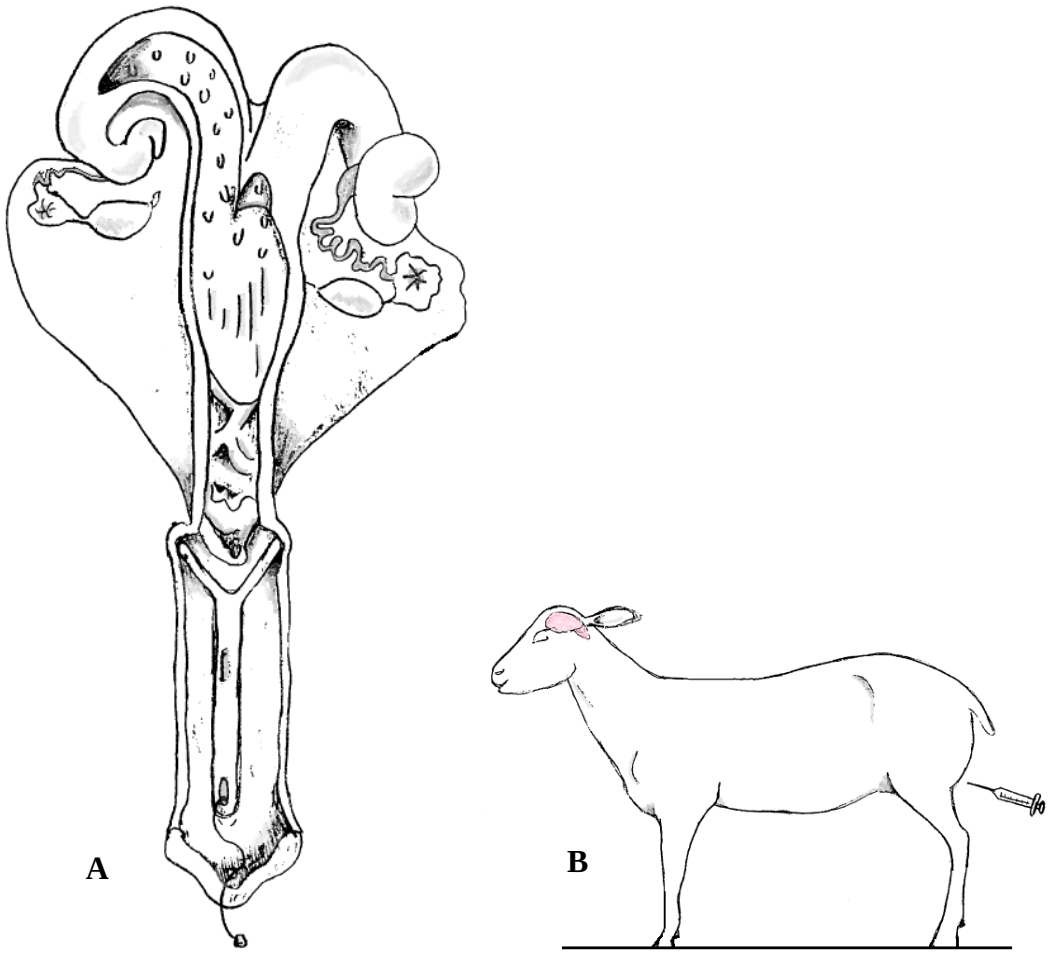

Fig. 3. A CIDR device inserted in the ewe vaginal canal (**A**). A Dinoprost tromethamine (PG) i.m. injection administration (**B**). Upper female reproductive tract image is modified from the book of Schatten and Constantinescu (2007) and lower part is an original drawing.

4. Materials and Methods

4.1. Animal Management

A total of 40 non-lactating, 2-4 years old crossbreed Barbados Blackbelly x White Virgin Island ewes, with a mean weight of 30.4 kg and a mean body condition score (BCS) of 2.9 out of 5, were treated in this study. Females were maintained in a sheep pen of 100 m^2, with no visual or physical access to rams, in the Research Farm Station at Ross University (17.3° N, 62.7° W) in St. Kitts and Nevis. Ewes were fed daily with 100 g of concentrate per animal (17% Crude Protein) and had free access to elephant grass (*Penisetum purpureum*), mineral salts, water and shade.

4.2. Experimental Design

Ewes were separated in two groups, at random stages of their estrous cycle. In the CIDR+PG group (n = 20), ewes were treated with a CIDR device (EAZY BREED-CIDR, Zoetis, USA) containing 0.3 g of P_4 for a 7-day period. After removal of the CIDR device, ewes were treated with one injection of 10 mg (2 mL) of PG analogue via i.m. (Lutalyse, dinoprost tromethamine, Zoetis, USA). For the PG group (n = 20), ewes were treated with two injections of 10 mg (2 mL) of PG via i.m., with a 10-day interval. This investigation is similar as described by the Ethiopia research conducted by Rekik (2016) but with an interval of 10 days instead of 11 days. Ultrasounds proceeded daily for 21 days after the last hormone administration in both groups. When no ovulation occurred in the first 100 h post-treatment, ewes were removed from the experiment and their P_4 concentrations and ovarian activity data were excluded from further analysis.

Evaluation of data was done for 16 animals to the CIDR+PG group, and for 18 animals to the PG group.

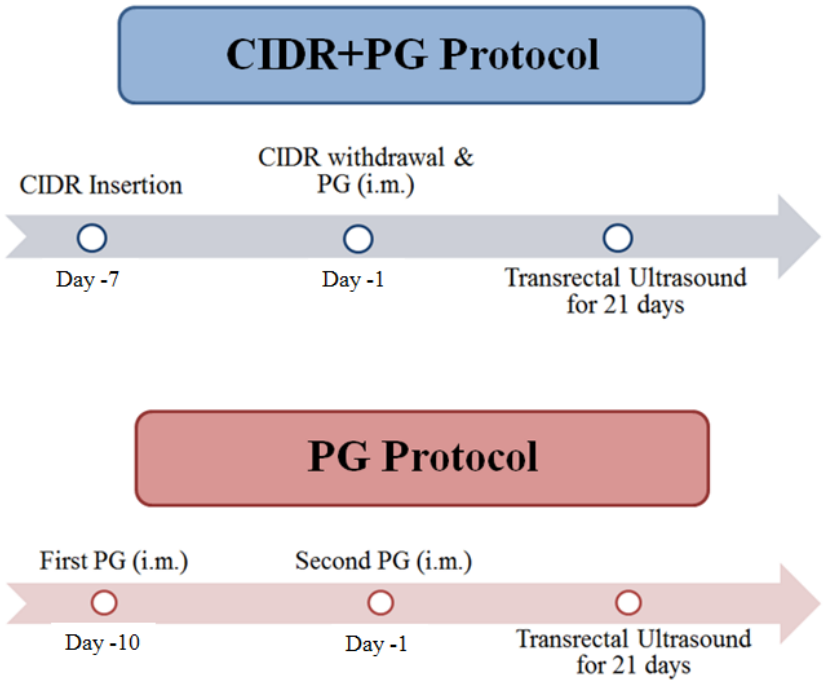

Fig. 4. Experimental designs of protocols, and both protocols started with 20 animals. The CIDR+PG protocol consisted in the insertion of a CIDR device for 7 days, later an i.m. injection of PG at CIDR device removal. The PG protocol consisted of i.m. injections of PG in a 10-day interval.

4.3. Follicular and Luteal Ultrasound Analysis

After each treatment, the ovaries of the ewes were examined once daily at the same daytime in the morning, using a B-mode transrectal ultrasound with a 7.5 MHz lineal array transducer (Honda HS-2200UV, MA, USA), for 21 consecutive days. The transrectal ultrasound technique was validated for the efficiency in monitoring ovarian

follicular and luteal development (Duggavathi et al., 2005; Ravindra et al., 1994). In every examination, a mapping of the ovaries including positions of luteal and follicular structures with measurements, were sketched in ovary schematic maps. With these ovarian charts, it was possible to analyze the pattern of development and regression of the follicles and corpus luteum in each ovary.

The interval (from treatment to ovulation) was considered as the time from the CIDR device removal, for the CIDR+PG group, and from the second PG administration, for the PG group. Ovulation was defined as the disappearance of a dominant follicle ($\geq$ 4 mm in diameter), that was detected during the previous examination (Day 0 = ovulation). Several ovarian activity parameters were monitored and studied, such as: the (1) ovulation rate, (2) mean interval of end of treatment to ovulation, (3) number of follicles ovulated, (4) mean diameter of pre-ovulatory follicle, (5) mean time of CL detection, (6) mean area of CL and (7) mean inter-ovulatory interval.

4.4. Progesterone Hormonal Analysis

Blood samples were collected daily by jugular venipuncture, using vacuum blood tubes with Heparin (Covidien Monoject™), from all ewes that were subjected to ultrasound examinations (n = 16, 18, CIDR+PG and PG group, respectively), for 21 consecutive days. The first blood sample was taken post injection of PG in the CIDR+PG group, and second injection of PG, in the PG group. Blood plasma was separated by centrifugation at 1500 x g for 10 min and stored at -20°C until assayed. P_4 concentrations were measured in plasma using an Enzyme-linked Immunoabsorbent

Assay (ELISA) kit (Arbor Assay DetectX, Michigan, USA) with 47.9 pg/mL sensitivity and with a 5.06% intra-assay coefficient of variation. When P_4 concentration in blood plasma is found 0.5 ng/mL or higher, then the ovulation process in ewes has been considered (Rekik, 2016).

4.5. Statistical Analysis

All data were presented in mean ± SEM and analyzed using SAS (Statistical Analysis System Institute Inc., Cary, NC, USA). Proportional and single-point measurements (i.e. pre-ovulatory follicle size, maximum CL diameter, ovulation rates) were compared among groups, and were analyzed using Chi-Square and Student t-Test. For repeated measurements, the one way ANOVA was done, and data (CL diameter, CL area and P_4 concentrations) were normalized to the Day of ovulation (Day 0) (Ali et al, 2006; Barret et al., 2004). The CL area was calculated by using the equation πr^2, eliminating the surface of the central cavity of CL, if present in the structure (Contrera-Solis et al., 2009). Significance was set at $p < 0.05$ (Ali et al., 2009; Oliveira et al., 2016).

5. Results

There were a percentage of animals that ovulated for synchronization protocols CIDR+PG and PG ($p > 0.60$) with an 80% and 90%, respectively (Table 1). However, there is a tendency towards a greater CL diameter ($p = 0.07$) and CL area ($p = 0.06$) in the CIDR+PG than in the PG group. Also, the duration of the inter-ovulatory interval was not affected by the protocol ($p > 0.70$), with an average of 16.4 ± 0.2 and 16.2 ± 0.2 d for CIDR+PG and PG alone, respectively. At Day 0 (ovulation), no CL was present and its detection occurred on Day 1 in both groups ($p > 0.80$). The day when the maximum CL size was observed, also did not differ ($p = 0.50$) between groups. Approximately by Day 9, regression of the CL was observed in both groups (Fig. 5). The average diameter of CL found in the CIDR+PG group tended ($p = 0.07$) to be larger than in the PG group, with a maximum growth of 9.9 ± 0.3 and 9.3 ± 0.1 mm, respectively (Table 1). The calculated CL area also tended to differ ($p = 0.06$), with an average maximum CL area of 78.1 ± 5.0 and 68.7 ± 2.4 mm² for the CIDR+PG and PG group, respectively. The CL diameter decreased to a minimum size in a similar way in both experimental groups ($p = 0.40$) before the next ovulation on Day 16, with CIDR+PG and the PG groups presenting 5.1 ± 0.1 and 5.5 ± 0.2 mm, respectively. From Day 0-7 post-ovulation, there were no differences in CL area between treatment groups ($p = 0.50$). The CL area in both groups exhibited an interaction ($p < 0.01$) from Day 8 to Day 16, where CIDR+PG CL area increased in size, while in the PG group it decreased in size. With the CIDR+PG presented a maximum difference of 13.49 mm² greater CL area from the PG group.

Table 1: Follicular and Luteal parameters in ewes treated with CIDR+PG or PG; (mean ± SEM).

	Treatment Groups		
	CIDR+PG (n=20)	**PG (n=20)**	**p Value**
Ovulation rate[a]	16/20 (80 %)	18/20 (90 %)	0.6
Interval from treatment to ovulation (h)	94.5 ± 4.6	98.7 ± 6.4	0.6
Number of ovulated follicles	1.6 ± 0.1	1.8 ± 0.1	0.1
Double Ovulation[b]	9/16 (56.2%)	14/18 (77.7%)	0.2
Number of CL	1.6 ± 0.1	1.8 ± 0.1	0.1
Size of preovulatory follicle (mm)	5.0 ± 0.2	4.8 ± 0.2	0.4
Inter-ovulatory interval (d)	16.4 ± 0.2	16.2 ± 0.2	0.7
Day of CL detection (d)	1.0 ± 0.2	1.0 ± 0.1	0.8
Day of maximum size of CL (d)	8.7 ± 0.6	9.2 ± 0.5	0.5
CL diameter by Day 16 of treatment (mm)	5.1 ± 0.1	5.5 ± 0.2	0.4
Maximum CL diameter (mm)	9.9 ± 0.3	9.3 ± 0.1	0.07
Maximum CL area (mm^2)	79.1 ± 5.0	68.7 ± 2.4	0.06

Note: Ovulation rate[a] and the incidence of double ovulation[b] are presented as the amount of ewes that ovulated over the total amount of ewes evaluated. The respective percentage values are presented in parenthesis.

The presence of a functional CL was confirmed when P_4 concentrations were greater than 0.5 ng/mL. The plasma P_4 concentrations increased from Day 1 through Day 6 in the PG group ($p > 0.05$), and continued to increase until Day 11 in the CIDR+PG group ($p < 0.001$) during the estrous cycle (Fig. 6). There was an interaction ($p < 0.0001$) between treatment and day, when the P_4 concentrations for the PG group increased and for the CIDR+PG group in decreased, from Day 7 until Day 16 (Fig. 6). In both groups, plasma P_4 concentration increased sharply to the maximum mean peak values of 12.75 ±

2.26 and 17.36 ± 1.61 ng/mL ($p < 0.001$), respectively. However, on Day 11 post-ovulation, a maximum P_4 concentration difference where the PG group presented 15.95 ng/mL and 1.41 ng/mL in the CIDR+PG group on the same day ($p < 0.0001$). It can be observed that a persistent decrease in the concentration of P_4 occurred until Day 13 in both groups ($p < 0.0001$), then it maintained a constant frequency up until the next detected ovulation.

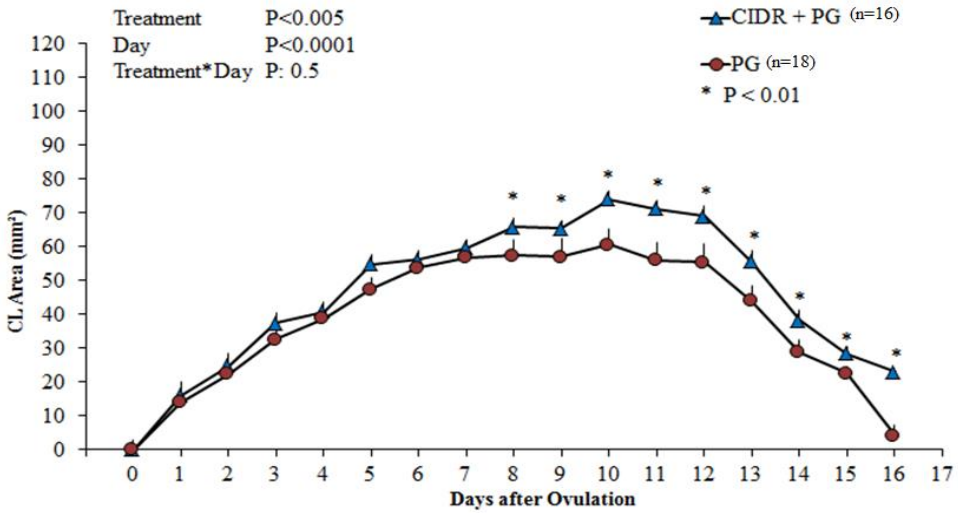

Fig. 5. Mean (± SEM) of single and double ovulations CL area (mm²) in ewes synchronized with CIDR+PG or PG. (Day 0 = Ovulation).

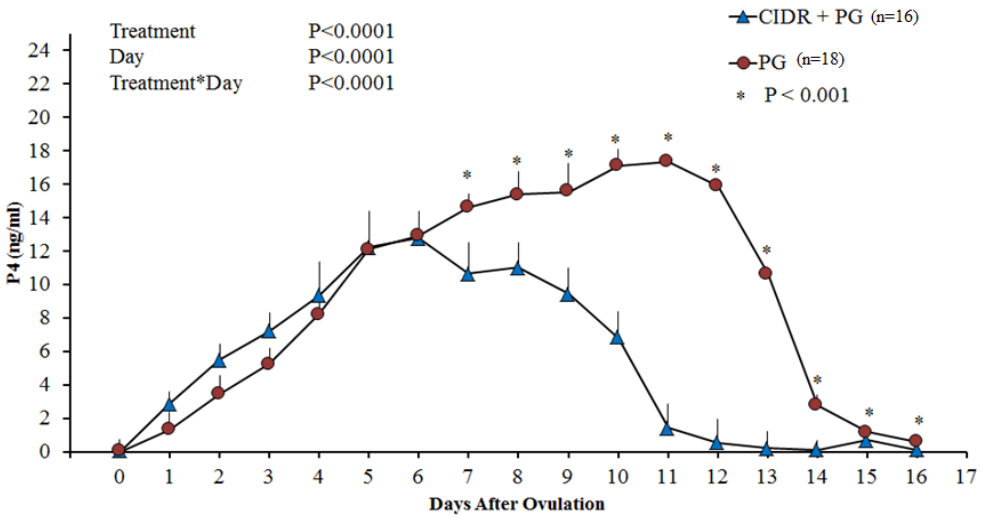

Fig. 6. Mean (± SEM) of single and double ovulations plasma P_4 concentration (ng/mL) in ewes synchronized with CIDR+PG or PG alone. (Day 0 = Ovulation).

There was a difference response in the P_4 concentrations found in the bloodstream in both groups ($p < 0.0001$). A perceptible decrease of plasma P_4 concentrations and an increase of the CL (mm^2) area in the CIDR+PG group demonstrated a contrary physiological response when compared to the PG group. The PG group showed an increase of P_4 concentrations and CL area in a parallel way. The CIDR+PG group had (Fig. 7) the highest P_4 plasma concentration mean of 12.75 ± 2.26 ng/mL with an average CL area of 56.16 mm^2. There was a sharp decrease of P_4 concentration by Day 8 (Fig. 7), even when the CL area remained a constant size until regression by Day 11 in the CIDR+PG group. The PG group presented its highest mean plasma P_4 concentration of 17.36 ± 1.61 ng/mL with an average CL area of 55.94 mm^2. The maximum P_4 concentrations and CL area happened at the same time for the PG protocol only.

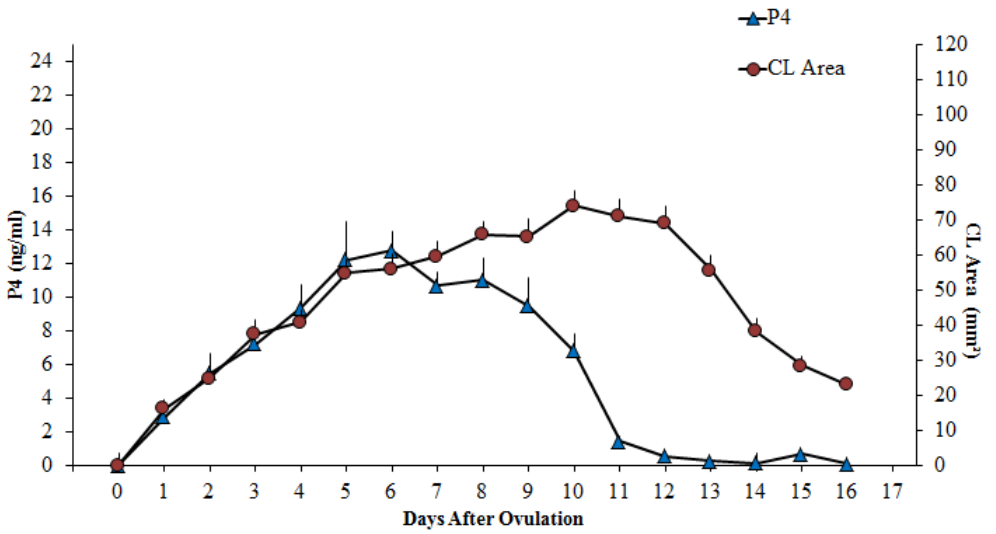

Fig. 7. Mean (± SEM) of single and double ovulations plasma P_4 concentration (ng/mL) and CL area (mm²) in ewes (n= 16) synchronized with CIDR+PG (Day 0 = Ovulation).

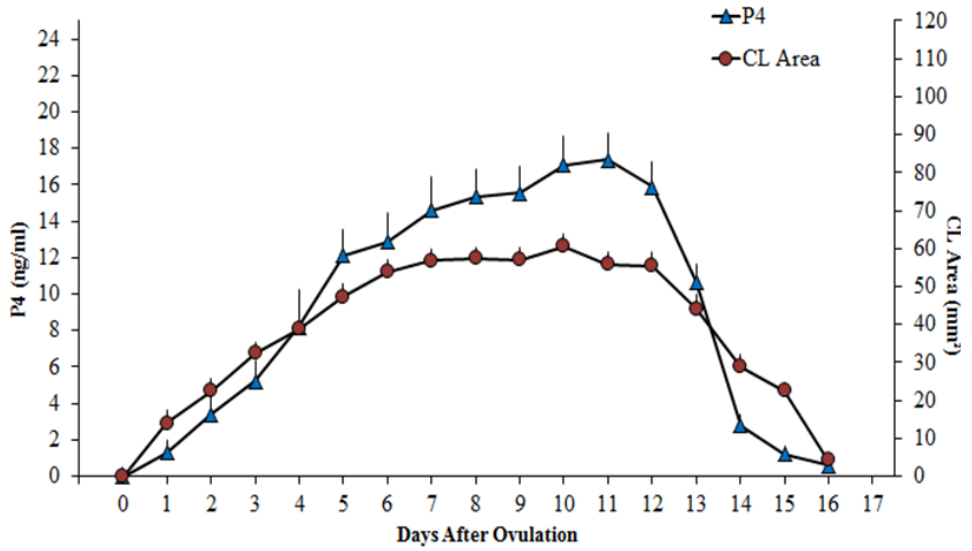

Fig. 8. Mean (± SEM) of single and double ovulations plasma P_4 concentration (ng/mL) and CL area (mm²) in ewes (n= 18) synchronized with PG (Day 0 = Ovulation).

6. Discussion

In this study, the hair sheep in tropical conditions responded well to the protocols by presenting high ovulation rates after finalizing synchronization treatments. The pre-ovulatory follicles were not significantly different in size and the surrounding follicles (non-dominant follicles, data not included) had a parallel smaller diameter compared to the dominant follicle, indicating a similar LH pulse frequency occurred in both groups (Dias et al., 2015). Although, a numerically higher number of double ovulations (14/18) was observed in the PG group, there were no differences with the CIDR+PG group (9/16) ($p = 0.20$).

However, the time for ovulation to take place was not consistent with the results obtained from other studies evaluating new and re-used intravaginal devices with ovulations at 60-70 h (Vilariño et al., 2013). Although, the research work performed by Carlson (1989) and Jackson (2014), presented a similar response to the CIDR+PG protocol, an interval ovulation time of 60-108 h was observed. This variability could be cause by the time needed for the maturation and growth of the pre-ovulatory follicles to take place (Campbell et al., 1995). For the PG protocol, if a growing follicle was present at the time of treatment, the follicle continues its development, and estrus and ovulation occur shortly after treatment (Loubser & Van Niekerk, 1981; Viñoles & Rubianes, 1998), as seen in the ewes treated in our study. This confirmed that the sheep in St. Kitts present this type of behavior, needing longer hours until ovulation, compared to other breeds at different environmental conditions. Even if the onset of ovulation took more hours, the inter-ovulatory interval of 16 days was very similar to the one reported in the other

investigation (Contreras-Solis et al., 2008), indicating that the estrous cycle length of tropical breeds did not differ from the norm in the length of the estrous cycle seen in ewes found in different environmental conditions.

Furthermore, it can be considered that this inconsequential decrease of P_4 concentrations was influenced by the smaller number of CL present during the estrous cycle, as can be observed in Figure 6, for the CIDR+PG group. Even if the CIDR+PG group presented less presence of CL, the sharp decrease of P_4 cannot be explained with the evaluated parameters, since CL structures were present at the time P_4 concentrations reached 1.41 ng/mL by Day 11. Also these animals, the CIDR+PG group, did not present estrus before the estrous cycle time frame of 16-17 days. According to Houghton et al. (1995), the CL should reach a mature stage of development, which involves its full endocrine functionality, to then start regressing. However, other investigation it was implied that CL size can be independent of the P_4 production in the luteal phase; indicating it could be influenced by other factors (Bartlewski et al., 1999a).

There could be a possibility that abnormal development of the dominant follicle occurred resulting in a non-functional CL, since these animals received high concentrations of P_4 for an extended period (7 days) (Mesen & Young, 2016). Another theory that can explain this demeanor relates to the fact that during the elevated exposure to P_4, the endometrium receptivity was affected and the exogenous P_4 induced an abnormal response when follicle development was taking place in the ewe (Mesen & Young, 2016). Further investigations to verify conception rates with these protocols could explain if the present corpus luteum had a complete luteal function. It can be

concluded that ewes in tropical conditions did respond to applied protocols, by entering estrus in a similar manner after protocol synchronization finalized.

7. Conclusions

The study showed that the evaluated synchronization protocols were efficient for estrus synchronization in ewes under tropical conditions. Both protocols produced approximately the same amount of double ovulations, confirming their natural tendency to be multiparous. In conclusion, application of CIDR+PG to ewes under tropical conditions resulted in a high degree of estrus synchronization that enhanced the structural development of the CL, especially in those animals with single ovulation. Additionally, it seems that the PG protocol could be a preferred way of synchronizing estrus in ewes under these tropical conditions since CL structures presented elevated concentrations of P_4 during the midluteal phase of the estrous cycle; in comparison in the CIDR+PG group these concentrations of P_4 were non-existent during the same phase in the estrous cycle.

8. Recommendations

For future research, these protocols should be evaluated with the presence of rams at the time of the CIDR device removal and in the last administration of PG, to evaluate if the "ram effect" will have a significant influence in accelerating the time of ovulation of ewes in tropical conditions. Other types of synchronization protocols should be also evaluated in these ewes found in tropical conditions. It could be a possibility of having a better response to the P_4 levels from the animals during the midluteal estrus cycle, by administrating GnRH at the moment the CIDR was removed (Rekik et al., 2016). Also the cost per administration is lower, when it comes to the moment of implementing these protocols including PG or GnRH, when large amount of animals is in the field (Rekik et al., 2016).

Thus, conception and pregnancy rates should be evaluated to determine if the CLs have complete functionality. With this validation, CL quality can be confirmed by the embryonic survival, since this information is still unknown under tropical conditions in hair breed sheep.

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