Universidade de Trás-os-Montes e Alto Douro

Follicular dynamics during the non-reproductive season jennies of the *Miranda* donkey breed.

- Versão Final -

Dissertação de Mestrado em

Mestrado Integrado em Medicina Veterinária

Sara Ramalheira Martins

Orientador: Doutor Miguel Nuno Pinheiro Quaresma

Vila Real, janeiro 2017

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Declaração

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Título da dissertação de mestrado: Follicular dynamics during the non-reproductive season in *Miranda* donkey breed jennies.

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Ano de conclusão: 2017

Declaro que esta dissertação de mestrado é resultado da minha pesquisa pessoal e da orientação do meu supervisor. O seu conteúdo é original e todas as fontes consultadas estão devidamente mencionadas no texto e na bibliografia final. Declaro ainda que este trabalho não foi apresentado em nenhuma outra instituição para obtenção de qualquer grau académico.

Vila Real, 31 de janeiro de 2017

Sara Ramalheira Martins

“The man who has no imagination has no wings.”

Muhammad Ali

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Abstract

The *Miranda* donkey breed had its origin in the most northeast of the Portuguese country and it is considered in risk of extinction. Studies on this breed have been done towards its better characterization and protection.

It is common the use of mare studies on jennies. However, the increasing number of donkey studies shows that their reproductive characteristics are distinct. The breeding season is with no doubt the most studied reproductive period in mares and jennies. Less seasonal effects in jennies and the necessity of better breeding results, particularly in endangered breed, leads to the interest in studying the non-breeding season.

The follicular diameters existent in both ovaries of fifteen *Miranda* jennies were measured from September to April, during two consecutive years and the characterization of the follicular dynamics during this period was done. The less marked seasonality of jennies was confirmed. However, a higher rate of seasonal effects, than what was found in literature, was found among our study population. Results on basic characterization of cycle lengths agree with the available literature. When comparing to the breeding season, longer interovulatory intervals were found, as well as longer estrus lengths and larger ovulatory follicles at the onset of the breeding season and spring transition. Diestrus periods were shorter among the group of Miranda jennies that underwent periods of anestrus. Two of the fifteen jennies consistently presented longer diestrus during both breeding and non-breeding season. Anestrus periods lasted longer than what has been previously published. Three anovulatory follicles were recorded, two during spring transition and one during vernal transition – all in jennies that underwent an anestrus period.

Ovulations from the left ovary were more frequent. More follicles were measured in the right ovary than in the left one during the entire considered period of time. The jenny that presented a persistent CL was the one that presented less follicles per ovary per ultrasound examination.

When observing follicular profiles of consecutive waves, an “area of ambiguity” was found that did not allow the distinction between regressing and developing follicles.

Indications of lower ovarian activity and smaller follicular diameters during the non-breeding season were corroborated by considerable high rate of follicles under 18 mm in diameter and a higher rate of larger follicles during estrus periods.

Minor waves were significantly more frequent among jennies that underwent an anestrus period. The emergence of consecutive minor waves during anestrus was the most frequent pattern in this group of jennies. Among the remaining jennies, the most common wave pattern was the emergence of only one follicular wave with either one or two ovulatory follicles. The diameter of the largest follicles of minor waves was generally lower than the diameter at divergence of major waves. Also, mean maximum diameter of the largest follicles of major secondary waves was smaller than the maximum diameter of the largest follicles of major primary waves.

It was found the presence of both major and minor secondary waves in all groups of jennies. Jennies that did not present an anestrus period presented, though not significantly, a higher number of follicular waves per cycle. Multiple ovulations with the ovulatory follicles being originated from different follicular waves were also found.

Key words: Jennies, *Miranda* donkey breed, non-breeding season, follicular waves

Resumo

A raça Asinina de *Miranda* teve a sua origem na zona do planalto Mirandês, no território mais a nordeste de Portugal. Esta raça encontra-se atualmente na lista de raças autóctones em risco de extinção pelo que estudos têm vindo a ser realizados no sentido da sua melhor caracterização e proteção.

É comum a aplicação de estudos realizados em éguas a burras, no entanto, o gradual aumento de estudo em asininos tem vindo a demonstrar que, nomeadamente a nível reprodutivo, existe um considerável número de diferenças. A época reprodutiva é sem dúvida o principal alvo de estudo da maior parte dos trabalhos publicados. Observações como a sazonalidade menos marcada das burras e a necessidade de otimização dos resultados reprodutivos, particularmente de espécies em risco de extinção, leva ao interesse no estudo da época não reprodutiva.

Este estudo visou a medição dos diâmetros foliculares presentes em ambos os ovários de quinze burras mirandesas durante os meses de setembro a abril durante dois anos consecutivos e a caracterização da dinâmica folicular durante este período. Confirmou-se a sazonalidade menos marcada das burras. No entanto, foi observado um maior número de animais que demonstraram efeitos sazonais que o publicado em estudos anteriores. Os resultados da caracterização dos períodos interovulatórios concordou com a bibliografia disponível. Quando comparados com a época reprodutiva, os ciclos apresentaram-se mais longos, assim como os períodos de estro e os folículos ovulatórios atingiram maiores dimensões no início da época reprodutiva e na época de transição da primavera. As burras que passaram por um período de anestro foram também os animais que registaram diestros mais curtos. Duas das 15 burras a estudo apresentaram diestros sistematicamente mais longos. Os períodos de anestro registados foram mais longos que o publicado em estudos anteriores. Foram registados três folículos anovulatórios, todos em burras que passaram por um período de anestro (dois ocorreram durante a época de transição de primavera e um durante a época de transição de outono.

As ovulações no ovário esquerdo foram mais frequentes. Foi medido um maior número de folículos no ovário direito em todas as burras ao longo de todo o período de estudo em todas

as ecografias realizadas. A burra que apresentou um corpo lúteo persistente foi também a que apresentou um menor número de medições por ecografia.

Ao analisar os perfis foliculares, foi encontrada uma área de ambiguidade que não permitiu a distinção entre folículos em regressão de folículos em crescimento.

Um menor nível de atividade ovárica e menores dimensões foliculares parecem ter estado presentes já que foi encontrada uma grande percentagem de folículos menores que 18 mm e uma grande concentração de folículos de maiores dimensões nos períodos de estro.

Ondas menores foram significativamente mais frequentes durante os períodos de anestro. A emergência de ondas menores consecutivas foi o padrão mais frequente em burras que entraram em anestro. Nas restantes burras a emergência de apenas uma onda com um ou dois folículos ovulatórios foi o padrão mais frequente. O diâmetro máximo do maior folículo de ondas menores foi inferior ao diâmetro à divergência dos maiores folículos das ondas maiores. O diâmetro máximo registado em ondas maiores secundárias foi inferior ao diâmetro máximo registado em ondas maiores primárias.

Foram identificadas ondas primárias e secundárias menores e maiores. Burras que não entraram em anestro apresentaram um maior, mas não significativamente maior, número de ondas foliculares. Verificou-se uma ovulação múltipla em que os folículos derivaram de duas ondas foliculares distintas.

Palavras-chave: Burras, Asinina de *Miranda*, Época não reprodutiva, Ondas foliculares

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Abbreviations

AEPGA	Associação Para O Estudo E Proteção Do Gado Asinino
AI	Artificial Insemination
FAO	Food And Agricultural Organization
BCS	Body Condition Score
CL	Corpus Luteum
COX-2	Cyclooxygenase-2
FSH	Follicle Stimulating Hormone
GNRH	Gonadotropin-Releasing Hormone
HCG	Human Chorionic Gonadotropin
IGF	Insulin-Like Growth Factor
IGFPB	Insulin-Like Growth Factor Binding Protein
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
MRNA	Messenger Ribonucleic Acid
P4	Progesterone
N/A	Not Applicable
PGF2A	Prostaglandin F2-Alpha
SD	Standard Deviation
US	Ultrasonography
VEGF	Vascular Endothelial Growth Factor

I. Introduction

1. The Donkey

Domestic donkeys (*Equus asinus*) and horses (*Equus caballus*) are the domestic representatives of the *Equus* genus, known to have some unique reproductive characteristics (Huang et al., 2015; Pugh, 2002). According to recent studies, all the existing equid species are grouped in a single genus – *Equus* and, under this taxonomy, the domestic donkey (*E. africanus asinus*) is considered a subspecies of the African wild ass (Rosenbom et al., 2015a). Archaeological findings have led to two different hypotheses about the origin of this species. Some state that the presence of donkey remains from 6000 to 5000 BC in Egypt is an indication of this species being domesticated by the people of Egypt in the Nile Valley; others locate the donkey's domestication in a northeastern African territory including the Sahara Desert 7000 to 65000 BC (Beja-Pereira et al., 2004; S. Rosenbom et al., 2015b; Scherf et al., 2015).

According to FAO, the donkey world population has increased from around 37 million donkeys in 1961 to around 44 million in 20014, with significant differences between regions; being the semi-arid zones the ones where the donkey concentration is higher. China and Ethiopia are the countries with larger numbers of donkeys (eleven and five million respectively) (Fielding and Starkey, 2004). There has been an increase by 60% in donkey populations in Africa, again with an uneven distribution among the countries in this continent. Latin America also shows an increase in donkey population. Central America has a small and steady growing donkey population. In South America there is a general increasing tendency, with the exception of Argentina and Chile where the populations have been decreasing. At last, in North America, the United States of America have always presented low numbers of donkeys that haven't increased significantly. Asia in general, Turkey, Iraq, Israel, Jordan and Lebanon have experienced a decrease in donkey populations (with the last three countries suffering little changes in the past decade). On the other hand, Iran, Pakistan and Afghanistan revealed a contrary tendency. There are also countries that maintained their donkey populations relatively stable (Saudi Arabia, Syria, Yemen and Oman). Oceania shows general low numbers of donkeys amongst its countries (Fielding and Starkey, 2004).

Pertaining to Europe, there are considerable differences from country to country, with the UK and Germany having small populations of donkeys and the Eastern European countries with a stable population tendency. France and Ireland presented major declines in the past decades, as well as some southern countries like Italy (with a decrease of 96% from 1939 to 1996), Spain and Greece. In Portugal, there has also been a great reduction in the number of donkeys. Between 1999 and 2009 the number of donkeys decreased by 60%, with 30% of all donkeys concentrated in the north of the country (Instituto Nacional Estatística, 2009). This tendency that some countries present in reducing donkeys population and number of breeds endangers World's domestic animal biodiversity (Aranguren-Méndez et al., 2002, 2001; Collins et al., 2012; Quaresma, 2014; Huang et al., 2015). A remarkable point is that a higher donkey population seems to be related to countries less industrialized, which had, and in some cases still have, agriculture as one of their most important economic activities.

2. The *Miranda* donkey breed

The *Miranda* donkey breed is a Portuguese donkey breed (Figure 1) that has its origin in the most northeast of the country, in *Planalto Mirandês*. As other Iberic breeds, Andalusian, Catalonian, Encartaciones, Mallorquina and Zamorano-Leones, it is facing some serious preservation issues (Aranguren-Méndez et al., 2002, 2001, Quaresma et al., 2014).

The specimens of this Portuguese breed are characterized for having a long brown bay coat, ideally around 130 cm of height and a calm temperament (Figure 2). These animals can be used for agricultural work, leisure, mediated therapy and milk production. This species milk is low fat, low protein and high lactose and can be used for both cosmetic and nutritional purposes (Muehlhoff and FAO, 2013; Quaresma et al., 2014).

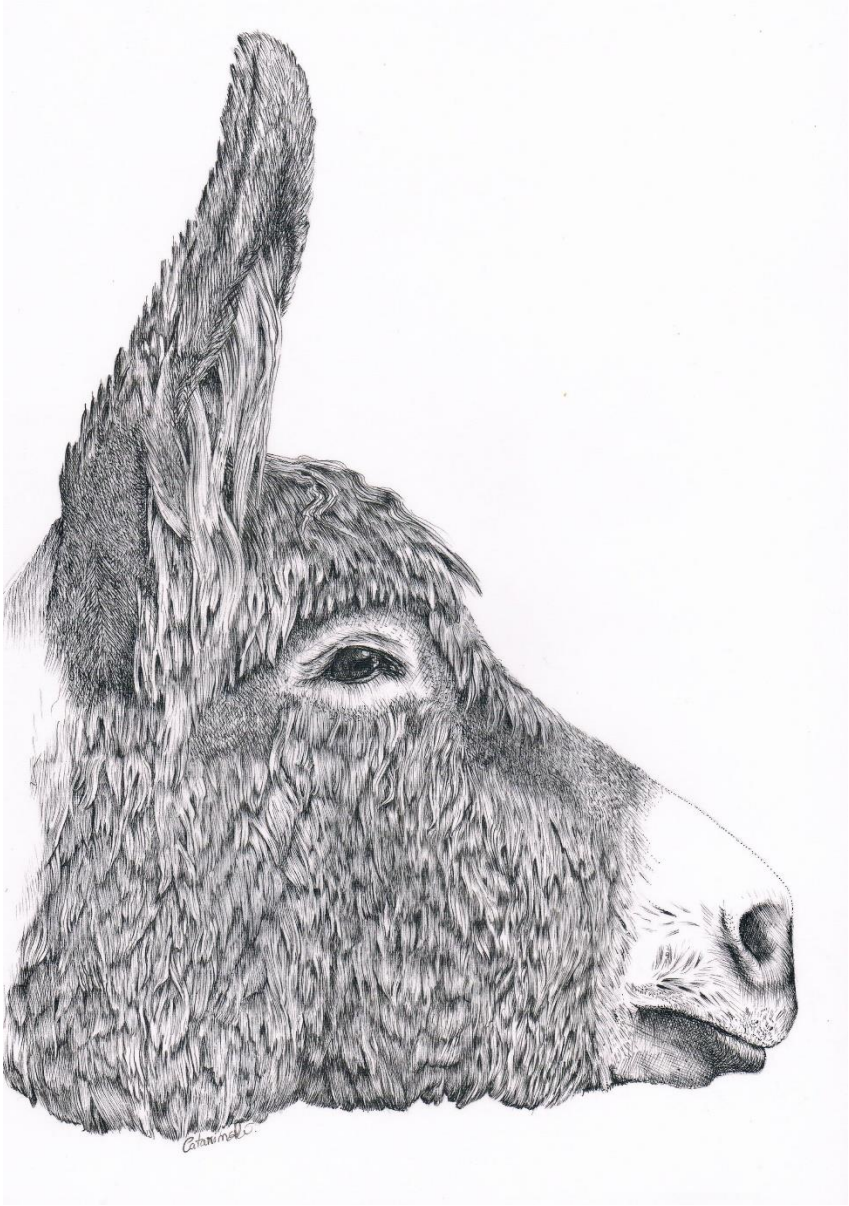

Figure 1 Profile illustration of a *Miranda* breed jenny in cross-hatching. Artist: Catarina Pereira, 2016.

Coming from an area with strong agricultural traditions, the *Miranda* donkey breed was mostly used by farmers to help with their daily workload (Beja-Pereira et al., 2004; Fielding and Starkey, 2004). Resembling many other industrialized countries, with the technological advances, the decreasing popularity of agriculture amongst young people, the emigration and the shifting in many traditions, the donkey has become less and less popular, with the *Miranda* donkey breed being considered in risk of extinction (AEPGA, 2016).

Figure 2 Reproductive exam by ultrasound examination on a *Miranda* donkey breed jenny *(Photo courtesy of Miguel Quaresma)*

The *Miranda* donkey breed studbook comprises 760 animals with a female:male ratio of 9:1. Even though the authors didn't find many consanguineous animals, the very low number of females and males available for reproduction, the aging of those animals and of their owners, the low breeding rate and the unequal contribution of the herds to the genetic pool are major concerns (Quaresma et al., 2014). Producing scientific knowledge that can help to implement good reproductive strategies is important for breed preservation (Aranguren-Méndez et al., 2002, 2001; Collins et al., 2012). Also, the *Miranda* donkey breed donkey is a versatile breed that besides agriculture, can still be used as a companion animal, for tourism, asinotherapy and their milk used for cosmetics (Pugh, 2002; Quaresma, 2014).

3. The estrous cycle of the jenny and the mare as a study model

The donkey reproductive cycle is similar in many ways to the horse, but has also some key differences (Huang et al., 2015). Being by far the most studied Equid, the mare is a starting point to obtain more information about the jenny (or jennet) and also other monovulatory species, including humans (Ginther et al., 2005a, 2004b; Mihm and Evans, 2008; Donadeu and Pedersen, 2008; Ginther, 2012).

3.1. Reproductive activity and cycle length

It is known that horses and, to a certain degree also donkeys, are long day breeders. Less domesticated horse breeds and pony mares usually demonstrate reproductive activity from May to October in opposition to more domesticated horses that tend to maintain their reproductive activity even in the winter season (Ginther et al., 1987; Aurich, 2011). On the other side, donkeys tend to show longer breeding seasons and a less marked seasonal anestrus, being most active between the months of March and August, sometimes continuing to ovulate during the rest of the year (Ginther et al., 1987; Pugh, 2002; Quaresma and Payan-Carreira, 2015).

Jennies usually achieve puberty between 1 to 2 years old, being the onset of puberty the result of many related factors such as photoperiod, temperature, individual health status, its BCS and genotype (Fielding, 1988; Pugh, 2002).

The estrous cycle of the donkey is longer than the mares' (Pugh, 2002). In 1981, a study conducted in Wisconsin with unknown donkey breeds, established a 24.9 days interovulatory interval with a corresponding 6.4 days estrus and a diestrus of 19.3 days (Vandeplassche et al., 1981). Still, only slightly different values were found in more recent studies for distinct donkey breeds and populations' interovulatory interval length: 24.9 days in the Catalan breed (Taberner et al., 2008) or 23.3 days in Mammoth Asses (Blanchard et al., 1999), 24.25 in Egyptian jennies (Derar and Hussein, 2011), 25.1 in the Tropical jenny (Lemma et al., 2006b) and 23.8 days for *Miranda* donkey breed jennies (Quaresma and Payan-Carreira, 2015), with indications of the age influencing the length between ovulations. Ovulations occur approximately 15 hours before the end of behavioral estrus, with the persistence of estrous behavior for a variable period of time. (Lemma et al., 2006c; Quaresma and Payan-Carreira, 2015), as for the horse.

During the breeding season, Quaresma and Payan-Carreira (2015) reported for the *Miranda* donkey breed an estrus length of 6.65 days and a diestrus length of approximately 17.9 days. Similar values were found for Catalonian jennies (5.64 days and 19.83 days for estrus and diestrus, respectively) and for Mammoth Asses jennies (5.9 and 17.4 days for estrus and diestrus length, respectively) (Blanchard et al., 1999; Taberner et al., 2008).

3.2. Reproductive behavior

During the period in which the mare is receptive to the stallion, behavioral routines tend to differ from the not receptive period. The time spent eating and resting diminishes and she becomes more active, ending up having some typical estrus behaviors (Clayton et al., 1981). There are three categories in which the reproductive courtship behaviors can be placed: attractivity, proceptivity and receptivity. The first stated category is measured by the behavior of the stallion towards the mare and can be shortly explained as the mare's value as a sexual stimulus, being involved posturing and olfactory signals, for instance. Proceptivity, in turn, refers to the mare's response to certain behaviors of the stallion such as vocalizations. At last, receptivity, refers to everything in the mare's behavior that allows the copulation with the stallion (Beach, 1976).

When more than one jenny is in estrus, they tend to stay together forming a kind of cluster, vocalizing together towards the jack. When the male donkey is teasing one jenny, usually the others stay nearby. McDonnell (1998) compared the sexual behavior of donkeys and ponies and concluded that the jack spends more time teasing the jenny, necessarily taking longer to reach an erection (mounting the jenny multiple times before reaching the erection) when handled than when left to run loose. The same authors and Clayton et al. (1981) also refer that the matting is usually longer in donkeys than in ponies.

Mouth clapping, mouth opening and closing, with the lips relaxed and the head and neck lowered and extended, also known as mouth champing, jawing or yawning, is considered the main behavioral sign of estrus in donkeys (Vandeplassche et al., 1981). Winking and tail raising (Figure 3) were also found to consistently appear among jennies. Mouth clapping is the behavioral sign that appears sooner and it is the last one to disappear. Mouth clapping produces a very characteristic sound audible by humans. Also described in donkeys, are ears back against the neck, hind legs splayed, one foreleg slightly back and the other slightly forward, presentation of the perineum to the jack and urinating small amounts of urine at a time (Vandeplassche et al., 1981; Fielding, 1988; McDonnell, 1998; Pugh, 2002).

Figure 3 *Miranda* breed jenny in estrus and evidencing mouth clapping behavior and with her tail raised *(Photo courtesy of Miguel Quaresma)*

Other two categories that can be established for reproductive behaviors (and where we can easily fit the behaviors listed above) are the homotypical and heterotypical signs of estrous; the first ones consisting in signs that are distinctive of a certain species, such as demonstration of interest towards the male, posturing, clitoral winking and mouth clapping for the donkey species. As heterotypical signs, there can be found animals sniffing or chasing other females, standing to be mounted or Flehmen responses (McDonnell, 1998). Studies conducted in the *Miranda* donkey breed (Quaresma and Payan-Carreira, 2015), Catalonian jennies (Taberner et al., 2008) and a group of jennies in Ethiopia (Kebede et al., 2012) registered similar homotypical and heterotypical estrus signs as the ones already described.

Taberner et al. (2008) also characterized the jacks' behavior towards the females in estrus and agreed with the previously described by Clayton et al. (1981) and McDonnell (1998): the jack vocalized and mounted the mare several times without erection, bit the neck, head and ends of the female donkey, smelt the perineal area and presented the Flehmen response. Also described is the jack covering of the jennies' excrements with urine (McDonnell, 1998).

During diestrus, jennies usually run away from the jack and can even try to bite or kick them to keep them away while keeping their tail closely to the perineum. Curiously, this running

behavior has also been described during the estrus phase but ending with the female allowing the jack to mount her (Clayton et al., 1981; McDonnell, 1998).

4. Endocrine Regulation of the estrous cycle

4.1. Hypothalamic and pituitary function

The hypothalamus is fundamental in the control of reproductive function in mares, by secretion of gonadotropin-releasing hormone (GnRH), a decapeptide which regulates the production and secretion of two other hormones: follicle-stimulating hormone (FSH) and luteinizing-hormone (LH). Initially called luteinizing hormone-releasing hormone (LHRH), its name changed when GnRH was found to interfere also with the dynamics of FSH in some species. In the horse, GnRH pulses are followed by LH pulses from the pituitary gland, generally together with a FSH pulse. The frequency of these pulses varies depending on which point in the estrous cycle the mare is. This pulsatile GnRH release is mainly controlled by feedback mechanisms. Structurally similar, LH and FSH, in conjunction, have an important role in fertility of both females and males. Both hormones are produced by a type of cells called gonadotropes from the pituitary gland. Together with thyroid-stimulating hormone (TSH) and equine chorionic gonadotropin (eCG), FSH and LH are part of the same glycoprotein family (Aurich, 2011; McKinnon, 2011).

In the horse, endogenous opioidergic systems are activated by progesterone and estradiol, so that in the luteal phase the hypothalamus is inhibited by these endogenous systems while during the follicular phase they are "inactive", allowing the pulsatile activity of the hypothalamus (Aurich, 2011; McKinnon, 2011). In the mare, a pronounced differential regulation of LH and FSH secretion is due to the existence of three types of gonadotroph cells in the *pars distalis* and *tuberalis* of the pituitary gland: monohormonal gonadotrophs of either FSH or LH and bihormonal gonadotrophs that store both hormones (Aurich, 2011).

4.2. Follicular development, follicular hormone production and ovulation

4.2.1. Mares

4.2.2.1 Follicular selection

During the mare's early fetal life, the primordial germ cells that have migrated, proliferated and become arrested during meiotic division at prophase I as primary oocytes, suffer atresia or develop into primordial follicles. At birth the ovaries contain thousands of these follicles, ready to develop into primary, secondary or antral follicles (Donadeu and Pedersen, 2008). The same phenomenon happens with all farm species (Driancourt, 2001). As in other species, as cattle or even in women, follicles develop in certain patterns as cohorts of follicles or follicular waves (Pierson and Ginther, 1987; Sirois et al., 1989; Bergfelt and Ginther, 1992).

Around day 7 of the estrous cycle of mares, being the day of the previous ovulation called day 0, a variable number of small antral follicles, with around 6 mm of diameter start to develop at a similar pace with less than a day of interval between emergence of consecutive follicles (Gastal et al., 2004) in what is called a follicular wave, together with a rise in the peripheral FSH concentrations. The period during which these follicles are recruited can be denominated "recruitment window" and can last 3 days in horses (Driancourt, 2001). In mares, it was reported that a mean of 12 follicles emerge per wave until deviation occurs, though it might vary; after deviation a mean of 4.5 follicles were reported to emerge occasionally until ovulation (Pierson and Ginther, 1987). At this point, several authors use the designation of common growth phase. Only gonadotropin-dependent follicles are recruited; gonadotropin-dependency develops when the follicle measures 2 mm in diameter; at this size is when they become. (Driancourt, 2001).

Follicular waves cannot develop without proper gonadotropin stimulation; a surge of circulating FSH precedes each follicular wave (Donadeu and Pedersen, 2008). It peaks about 3 days before the beginning of recruitment or when the largest follicle has around 13 mm of diameter (Irvine et al., 2000; Ginther et al., 2003a). Usually, the future dominant follicle emerges earlier than the remaining follicles, and maintains a 3 mm advantage from the second largest follicle, as the follicles grow at a similar pace at this point of the follicular wave

(Gastal et al., 1997, 2004). A similar situation occurs with cattle (Ginther et al., 2003a; Ginther et al., 2001b), but not with women (Baerwald, 2003; Baerwald et al., 2003).

Not only the FSH concentrations influence the developing follicles, but also follicles can influence the FSH concentrations, in this case negatively. It is believed that an increasing concentration of inhibin-A, secreted by granulosa cells and present in the follicular fluid, from mainly the largest follicles, that are around 13 mm at this point, is involved in the decrease of circulating concentrations of FSH (Watson and Al-Zi'abi, 2002a; Ginther et al., 2003b). Not only inhibin, but also androgens and estradiol might contribute to the FSH decline (Evans et al., 1997). Among these follicles, the future dominant follicle or follicles are emerging. Later, around day 13, follicle deviation occurs (Aurich, 2011).

Deviation can be considered a mechanism of monovular species (mares, cattle, women, etc.) to ensure the outcome of a single offspring, increasing this way the chances of survival in these species (Mihm and Evans, 2008). In the mare, deviation occurs when the dominant follicle has reached 21 to 23 mm and consists of a process that will prevent subordinate follicles from growing as much as the dominant (Ginther, 2000; Gastal et al., 2004; Beg and Ginther, 2006). The second largest follicle usually measures around 19 mm at this point of the cycle and might keep its capability to reach dominance for at least 1 day after the beginning of deviation in case the dominant follicle fails (Ginther et al., 2003b; Gastal et al., 2004). In some mares, follicles as small as D5 or D7 (being D1 the largest, D2 the second largest and so on) managed to reach dominance after ablation of the largest follicles, demonstrating that, in some way, the capacity to reach dominance is prevalent among all follicles of a wave (Gastal et al., 2004).

An interesting phenomenon happens at this point of the cycle. While levels of FSH have decreased and are insufficient to allow growth of all follicles of the wave, the reduced concentrations of this hormone are still enough to allow the largest follicle to keep its way into becoming a dominant and later on, an ovulatory follicle. This happens due to a rise in gonadotropin receptors by the dominant follicle. The concentrations of inhibin and estradiol have begun to rise 1 day before the beginning of deviation, around 10 days before ovulation (Ginther et al., 2003b; Ginther et al., 2008a) or at the day of deviation (Ginther et al., 2007e). Also, the dominant follicles' sensitivity to FSH and LH increases in part due to an increase in

its gonadotropin receptors in the theca, simulated by elevated intra follicular levels of estradiol (Donadeu and Pedersen, 2008). Ginther et al. (2003b) have demonstrated the appearance of an ultrasonographic anechoic layer in the future dominant follicle, which likely represents an increase in its vascularity leading to the hypothesis that the largest follicle is, in fact, the main source of the higher levels of estradiol around the time of deviation.

The rise in LH circulating concentration, two days before deviation, and the rise of follicular sensitivity to this and other hormones by the rise of its intra follicular receptors is very important (Ginther et al., 2007e). This, allows the largest follicle of the wave to become dominant and for the development of ovulatory competence, for instance, a full responsiveness to LH surges near ovulation (Goudet et al., 1999; Gastal et al., 2000; Ginther et al., 2003a; Donadeu and Pedersen, 2008). In other words, it can be said that the increase in the dominant follicle's dependency of on LH is critical for ovulation to occur (Gastal et al., 2000). However, there are still some authors that question whether the LH dependency is a cause or a consequence of follicle selection (Mihm and Evans, 2008).

Circulating estradiol concentrations have been reported to fluctuate depending on the phase of the estrous cycle in which the mare is (Gastal et al., 1999; Irvine et al., 2000). More recent studies achieved different conclusions, indicating that estradiol does not actually play a role in the onset of deviation in mares (Donadeu and Ginther, 2002a; Beg and Ginther, 2006). Additional hormones that behave in a similar way are testosterone and cortisol. Testosterone reaches its highest level the day before ovulation and decreases during the initial luteal phase, while cortisol presents itself in high concentrations during luteal phase and low during follicular phase (Ginther et al., 2007e).

Androgens and progestins are known to be direct and indirect substrates for estradiol production, respectively. Following LH stimulus, theca cells produce androgens that are later aromatized into estrogens in granulosa cells (Beg and Ginther, 2006). Also, androgens can enhance the production of progestins at the granulosa cells. These substances are however, probably not involved in the deviation mechanism due to the absence of a discrepancy in progesterone concentrations before and after deviation and between the largest and second largest follicles after the ablation of the first one (Donadeu and Ginther, 2002a; Ginther et al., 2002, 2007e).

Activins, like activin-A and follistatin, are present in the equine and bovine follicular fluid. They are different glycoproteins that bind with each other (Austin et al., 2001; Donadeu and Ginther, 2002a). Knight and Glister (2001) have proposed a list of situations were activin participates: 1) it induces granulosa cell proliferation, 2) increases FSH receptor expression, 3) boosts granulosa cell steroidogenesis, basal and gonadotropin stimulated aromatase activity, estradiol production and also 4) delays luteinization and atresia. Another distinctive action of activin is the block of LH and estradiol androgen secretion from theca cells – this is when follistatin, as an activin binding protein, stops this inhibitory effect (Knight and Glister, 2001; Wrathall and Knight, 1995).

Activin receptors are present in both thecal and granulosa cells (Knight and Glister, 2001). Different results were obtained among studies in cattle (Austin et al., 2001; Beg et al., 2002; Donadeu and Ginther, 2002a) though it is believed that only the largest follicle reaches a certain responsiveness to activin-A which might be involved in the changes of concentrations of estradiol and IGF-1. This glycoprotein was found to increase differentially between the largest follicle and the other follicles of the wave like estradiol, free IGF-1 and inhibin-A leading to the possibility of a role in the increased FSH responsiveness seen in the dominant follicle of the wave (Ginther et al., 2003b; Beg and Ginther, 2006).

The IGF system is involved in growth and differentiation of cells and comprehends IGF-1, IGF-2, IGF receptors, binding proteins (IGFBPs) and IGFBP proteases. A study conducted in mares points out the possibility of the IGF system being crucial for the deviation to occur, even though estradiol, inhibin-A and activin-A concentrations are similarly higher in the dominant follicle than in the remaining subordinates (Beg and Ginther, 2006). Actually, it was reported that after ablation of the largest follicle and before the beginning of deviation, the concentration of free IGF-1 improved at the second largest follicle when compared to the third largest follicle, and that the concentrations of estradiol, inhibin-A and activin-A only increased after deviation began (Ginther et al., 2002). The effects of a change in IGFBPs combined with an increase in IGF produced at the ovarian cells are the following: stimulation of granulosa cell proliferation and steroidogenesis and probably an interference in luteal function (Spicer and Echternkamp, 1995).

An IGF-1 treatment of the second largest follicle (F2) resulted in 81% of these follicles deboming dominant and 62% ovulating. Previously, IGF had been reported to influence the concentrations of activin-A, inhibin-A and VEGF during follicle selection in mares together with a reduction in the production of androstenedione (Ginther, 2003b). However, IGF-1 was concluded to be an intrafollicular factor that does not affect systemic concentrations of hormones like FSH, estradiol, inhibin or LH. Follicles of a certain diameter or development stage, after the beginning of deviation, might have capability to reach dominance but can't produce IGF-1 (Ginther et al., 2008b).

Vascular endothelial growth factor (VEGF) is an angiogenic factor that appears to be augmented in the largest follicle when compared to the second largest follicle of the follicular wave. The largest follicle presents a higher blood flow, allowing a better nutrient, hormone and growth factor supply (Mihm and Evans, 2008). Evidence of an increased follicular vascularization is suggested by the anechoic layer at the ultrasound images (El et al., 1999; Ginther, 2003b) and were confirmed later by Doppler studies in horses (Acosta, 2004a), but not in cattle (Acosta et al., 2005).

Genomic studies have been conducted in cows, though other monovular species, as humans and horses, have not been yet studied. Results indicate the presence of 18 different genes that are expressed differentially among the largest and second largest follicles and that are the differentiation genes and not others, until then, associated with bovine dominant follicle differentiation, estradiol production, anti-oxidant events, LH sensitivity, cell proliferation, anti-apoptotic activity and mRNA splicing genes (Evans, 2004; Mihm and Evans, 2008).

2.4.2.2. Ovulation

In mares, at day 17, the dominant follicle usually measures around 35 mm and continues to grow while the subordinate follicles begin to regress. At the beginning of the preovulatory stage there is a pronounced rise in follicular estradiol that only allows a mild rise in LH concentrations, accompanied by a discrete decrease in FSH that will eventually return to its basal levels. Estradiol peaks two days before ovulation. LH will later exert a negative effect on estradiol and on follicle growth and its concentration continues to raise at a more

pronounced rate. LH will be responsible for a decrease in estradiol concentrations, the final maturation of the dominant follicle and ovulation when the follicle reaches around 40mm of diameter. FSH will discreetly rise due to the end of the negative effect estradiol has on this hormone and LH (Ginther et al., 2008a).

Near the day of ovulation (day 21), an increase in LH concentration is noticeable occurring also the raise of progesterone levels. Differently from other species, in the mare, the period of elevated concentrations of LH lasts 1 to 2 days and does not assume the shape of a short and pronounced preovulatory peak but rather a plateau. The vascularization of the ovulatory follicle increases (Aurich, 2011).

As already mentioned, the estradiol concentration peaks 1 to 2 days before ovulation and some observations suggest that if the level of estradiol is not enough for a proper positive feedback, the LH surge would not occur (Irvine et al., 2000). This disagrees with other authors, that defend a negative feedback relationship between these hormones (Ginther et al., 2008a). Irvine et al. (2000) also states that the decrease in estradiol concentration probably occurs due to the luteinization of granulosa cells and to the absorption of estradiol by the follicular fluid and its discharge into the abdomen (Ginther et al., 2008a; Aurich, 2011; McKinnon, 2011). This is accompanied by a decrease in uterine edema, typically seen during estrus. Estradiol promotes physical (cervix relaxation, uterine edema and increase in uterine secretions), endocrine (stimulation of hypophyseal LH release) and behavioral changes in the mare (Aurich, 2011; McKinnon, 2011).

Inhibin is a hormone produced by the granulosa cells dominant follicles that reaches its peak at the day of ovulation and remains in low levels during diestrus (Irvine et al., 2000). Higher levels of this hormone are directly related to estradiol and inversely correlated with FSH concentrations. Actually, one acknowledged function is to diminish FSH secretion by the hypophysis, as previously mentioned (Aurich, 2011; McKinnon, 2011).

In regard to ovulation and the pre ovulatory follicle, the size of this follicle can assume a wide interval, still, it has been shown that there is a considerable repeatability within each animal; studying a sample of estrous cycles of a certain female, the average size of the preovulatory follicle becomes an important information to predict the time of ovulation (Cuervo-Arango

and Newcombe, 2008). Usually it varies from 40 to 45 mm in diameter, with a growth rate of approximately 3mm/day (Ginther et al., 2008a).

The preovulatory follicle experiences some morphological modifications that can be perceived by a B Mode or Doppler ultrasound exam (Ginther et al., 2007c). Follicle maturity appears to be characterized by the appearance of a serration of the granulosa; irregularities among this layer of cells are obvious between 12 to 1h before ovulation, probably being the most useful sign to anticipate the moment of ovulation. The presence of apoptotic cells among the granulosa layer is positively correlated with follicles near ovulation (Ginther et al., 2007c). Also, decreased turgidity, follicles' loss of spherical shape, reduced area at one end (apex) and the antrums' fluid becoming less anechogenic and appearing to have echoic spots are all signs to have in mind when evaluating a pre ovulatory follicle (Gastal et al., 2006; O.J. Ginther et al., 2007a).

The ovulatory follicle will cease its growth rate and reach a plateau close the diameter with which it will ovulate. It will then rupture at the ovulation fossa (Ginther et al., 2008a). The oocyte and *corona radiata* then enter the oviduct, while the majority of the follicular fluid end up at the peritoneal cavity where hormones will reach systemic circulation – justifying the inhibin increase at the day of ovulation (Bergfelt et al., 1991).

Multiple ovulations can be defined as more than one ovulation occurring in a single ovulatory period or estrous cycle. Also they can be unilateral if all follicles ovulate from the same ovary, whether the left or the right one, or bilateral if the ovulatory follicles come from both ovaries. This phenomenon can also be classified as synchronous – if all follicles ovulate within an interval not larger than 24h, or asynchronous if ovulation occurs among different days. Transretal real-time ultrasonography has shown that 85.9% were single ovulations and 14.1% were multiple ovulations. Moreover, 14 out of 27 double ovulations came from only one ovary (unilateral ovulations) and the remaining 13 were bilateral ovulations with no relevant differences in the number of synchronous and asynchronous ovulations. Another conclusion reached was that the diameter of the pre-ovulatory follicle was different between single (the largest ones), double unilateral and double bilateral ovulations (the smallest ones) (Ginther and Pierson, 1989). Differently from the percentages presented above it was reported a 43% incidence of multiple ovulations (Ginther et al., 2008c) and, in another study during the

same year an incidence of 40% of double ovulations in mares, 2% in ponies and 25% in thoroughbreds (Ginther et al., 2008a).

It is known that the occurrence of multiple ovulations is variable, depending on the breed and even on each animal. Some studies concluded to be a variable grade of repeatability of multiple ovulations within mares influenced by the reproductive status, the age and even on the drugs administered to the animal during the cycle (Ginther et al., 2008a; Aurich, 2011;).

Until the size of 30 mm, both single and double ovulating mares present dominant follicles with similar sizes, with the known discrepancies appearing in the 2.5 days after this moment probably due to differences in FSH concentrations consequence of higher estradiol concentrations present in double ovulating mares. Moreover, the cessation or reduction of the size of the ovulatory follicle near ovulation was observed in both dominant follicles in double ovulating mares (Ginther et al., 2008c). Concentration of LH is believed to be similar among single and double ovulating females until ovulation, when a higher concentration of progesterone coming from the presence of two CLs will lead to a lower concentration of LH (Ginther et al., 2008c).

4.2.2. Jennies

The information previously mentioned within this chapter was on the mares' reproduction, as unfortunately there aren't enough studies about the donkey species that can provide as much information as needed for a full description on follicular development and hormone fluctuations in equids. However, studies on donkeys' follicular and gonadotropin levels and dynamics were published and their conclusions will be now presented.

Vandeplassche et al. (1981) found to be a significant day effect for FSH and LH concentrations, for diameter of the largest follicle and the number of small, medium and large follicles. Besides, the first significant increase in diameter of the largest follicle takes place 7 days before ovulation. Similar results where later reported also in Egyptian jennies; with a mean maximum diameter of 36 mm, 1 day prior to ovulation (Derar and Hussein, 2011), smaller than what was later reported for Catalonian jennies (Taberner et al., 2008).

The ovulatory follicle of *Miranda* donkey breed jennies measures around 38.4 mm (Quaresma and Payan-Carreira, 2015), smaller than the mean diameter of 46.3 mm of those in Catalonian jennies (Taberner et al., 2008), 41 mm from a group of jennies studied in Egypt (Derar and Hussein, 2011) and 44 mm (maximum diameter registered) in a group of jennies from Ethiopia (Kebede et al., 2012) but higher than the ovulatory follicle of tropical jennies during the short rainy season (37.8 mm) (Lemma et al., 2006c) or Anatolian jennies (32.25 mm) (Kalender et al., 2012).

Also, the Portuguese breed of donkeys presents a greater growth rate after the onset of estrus (3.18 mm/day) than before the onset of estrus and presents a slowdown in its growth on the day before ovulation (Quaresma and Payan-Carreira, 2015) agreeing with what was published in 1981 by Vandeplassche et al. and with what was published for Catalonian jennies (Taberner et al., 2008). In Egypt a slower growth rate was observed in jennies, about 2.32 mm/day, for the same period (Derar and Hussein, 2011).

A study on tropical jennies in Ethiopia assessed the mean number of follicles per wave and found it to oscillate through seasons between 7.3, 9.6 and 11.3 for the dry, short rainy and long rainy seasons, respectively. A higher frequency of medium and large follicles during the short rainy season was observed (from March to May) (Lemma et al., 2006c). Also reported was a comparative abundance of medium follicles (Kebede et al., 2012); all this data presents itself to be slightly different from the results published for mares, emphasizing the differences these two species have, particularly in regard to seasonality (Pierson and Ginther, 1987; Gastal et al., 2004). Another report from Egyptian jennies refers a lower number of follicles per ovary than the ones quoted above, though it is hard to access if the difference in their results is real or influence of the method used to count the follicles (Abdoon et al., 2014).

Medium follicles start to appear at day 7 before ovulation and then their number decline at day 4 before ovulation reaching “0” after ovulation. As for the number of small follicles, it decreases significantly as ovulation day approaches (Vandeplassche et al., 1981).

Available data on the number of follicular waves per cycle reports the existence of 2 to 3 waves per cycle in jennies under controlled management in Ethiopia (Kebede et al., 2012), contrarily to a single wave per cycle reported in jennies from Egypt (Derar and Hussein, 2011).

Deviation take place near day 9 before ovulation in *Miranda* donkey breed jennies, when the largest follicle measures a mean of 19.18 mm (smaller in case of multiple ovulations) (Quaresma and Payan-Carreira, 2015), earlier than what was reported in jennies from Egypt; 5 to 6 days before ovulation the dominant follicle measures approximately 25 mm (Derar and Hussein, 2011).

The occurrence rate of single versus multiple (double or triple) ovulations is a contentious point among the estrous cycle of donkeys with different studies reaching different percentages. The previously quoted study of Vandeplassche et al., (1981), refers that only one double ovulation occurred in 1 out of 7 jennies, among all the estrous cycles that were studied, which reflects a low rate of multiple ovulations. However, this study was published before ultrasonography started to be associated with reproductive exams, which by itself can explain such different results from the more recently published. Also worth mentioning is the tendency that these authors found in multiple ovulations being repeatable within jennies, as previously mentioned for mares (Aurich, 2011) and confirmed in the *Miranda* donkey breed (Quaresma and Payan-Carreira, 2015) and Catalonian jennies (Taberner et al., 2008). Differently from donkeys, mules did not present multiple ovulations (Volpe et al., 2005).

For *Miranda* donkey breed jennies, Quaresma and Payan-Carreira (2015) reported single ovulations to be more common followed by double and triple ovulations respectively, which agrees with Vandplassche et al. (1981) results. In 33 cycles, 57.58% were single ovulations, 36.36% were double ovulations and the remaining 6.06% correspond to triple ovulations. In Catalonian jennies the numbers are quite similar with 55.66%, 42.45% and 1.89% of single, double and triple ovulations respectively (Taberner et al., 2008). Another study on Spanish donkey breeds, analyzed 258 estrous cycles and of these 49.2% corresponded to multiple ovulations (43.4% of double ovulations and the remaining 5.8% of triple ovulations, with a higher frequency of multiple ovulations at the beginning of the breeding season) (Galisteo and Perez-Marin, 2010). T.L. Blanchard et al. (1999) have reported higher rates than the presented above for Mammoth jennies, with 11 out of 18 jennies presenting multiple ovulations.

Ovulations from the right and left ovary assumed similar number of occurrences in *Miranda* jennies (Quaresma and Payan-Carreira, 2015) and in Catalonian jennies (Taberner et al., 2008), even though it was observed a slightly bigger number of ovulations from the left

ovary, it was not significant. Studies on Tropical jennies also agree with these observations (Lemma et al., 2006b).

FSH concentrations during the jenny estrous cycle were recorded to be significantly high from day 10 to 12 before ovulation (> 11,0 ng/ml) and minimum 3 days before ovulation (7,4 ng/ml), in general agreement with the previously quoted for mares (Vandeplassche et al., 1981; Donadeu and Pedersen, 2008). In a study that used a sample of 14 *Miranda* donkey breed jennies, serum concentrations of progesterone were measured throughout the estrous cycle. Progesterone remained in very low concentrations at 24h before ovulation and then after ovulations. P4 levels raised and remained high until day 15 after ovulation (luteal phase) to posteriorly start to decrease 2 to 3 days before the onset of estrus. Multiple ovulations were associated with higher concentrations of progesterone (Quaresma and Payan-Carreira, 2015). In relation to E2 levels, these were measured in Anatolian jennies and were at 3.78 ng/ml on the day of ovulation (Kalender et al., 2012).

4.3. Luteal function and posterior luteolysis

The equine corpus luteum (CL) presents itself usually pear-shaped and it is characterized by growing towards the inside of the ovary, contrary to what happens in the majority of other mammals (Kimura et al., 2005). Equine CL is formed by small and large luteal cells and non-luteal cells (fibroblasts, smooth muscle cells, macrophages and endothelial cells) all originated from follicular cells of the already ovulated follicle, as thecal cells are progressively substituted by fibroblasts after ovulation and within only 24h after ovulation they will all be in an advanced stage of degeneration (Aguilar et al., 2006; van Niekerk et al., 1975). Luteal and non-luteal cells will start to develop about 10h after ovulation and will later reach their maximum size (van Niekerk et al., 1975). Also, progesterone producing cells and angiogenic factors (VEGF) present a marked activity (Al-zi'abi et al., 2003; Aguilar et al., 2006).

The main hormones controling of the luteal phase are LH and progesterone, allowing CL function. In equids, progesterone concentrations start to increase right after ovulation, reaching maximum values 8 days after they began to rise and decreasing between 8 until day

14, when luteolysis ensues, with the expression of progesterone receptors in large luteal cells (da Costa et al., 2005; Aguilar et al., 2006). Anatolian jennies present a mean concentration of 0.81 ng/ml of at the day of ovulation P4 (Kalender et al., 2012) and *Miranda* donkey breed jennies present mean serum progesterone levels 24h after ovulation of 0.48 ± 0.14 ng/ml that reach 5.56 ± 0.86 ng/ml 72h after ovulation continuing to increase during mid luteal phase (Quaresma and Payan-Carreira, 2015). Some studies refer that maximum P4 concentrations reach values above 10 ng/ml (Quaresma and Payan-Carreira, 2015). Others, refer higher values as 27.72 ± 14.02 ng/ml at the 14th day after ovulation at the first cycle after parturition (Kalender et al., 2012). Kebede et al. (2012) verified that this hormone starts to drop at day 15 to 16 after ovulation and will be kept at its nadir until next ovulation occurs.

After ovulation, LH presents high concentrations, suggesting an important role in luteal development (Ginther, 1992). The dynamics between LH, progesterone and estradiol were studied in 9 mares during interovulatory period, and similar results were obtained with progesterone reaching its maximum concentration on day 6 after ovulation and decreasing after that interval (Ginther et al., 2006). P4 concentrations of about 2 ng/ml at the beginning and end of the luteal phase were responsible for negative effects on LH concentration, this way, justifying the progressive decrease in LH concentrations after ovulation (Ginther et al., 2008a). Mules present lower maximum concentrations of P4 (5 to 7 ng/ml) when comparing to the mare (Volpe et al., 2005).

It was also observed a decrease in concentrations of these hormones at a similar pace from day 6 until day 14 when luteolysis was expected to commence, consistent with declining positive effect of LH concentrations of progesterone. The area of the CL and plasma progesterone concentrations were compared, showing that they behaved in parallel throughout luteal phase but were asynchronous during the luteolytic period where CL area decreased at a lower rate than progesterone concentration (Ginther et al., 2007d).

Besides LH and Progesterone, IGF system is believed to be responsible as well for the control of the luteal phase, being the increased presence of IGFBP-2, most likely, a cause for luteolysis by inhibition of IGFs and their action on preventing apoptosis and stimulating steroidogenesis (Watson et al., 2005).

After day 14 of the cycle, the concentration of progesterone starts to decrease and, later, morphological changes of the CL will occur, with death of luteal cells. A decrease in the expression of $P450_{scc}$, a steroidogenic enzyme, was found, where it was also studied the expression of IGFBP-2 mRNA (Watson et al., 2005). VEGF will also decline but it is not believed to contribute in the apoptosis of the CL cells (Aguilar et al., 2006; Ginther et al., 2007d). A study that involved 18 mares, suggested that the developing follicles of a new follicular wave at the ovary might have a role in morphological regression of luteal structures, as they took longer to regress when follicles were ablated before they reached 10 mm in diameter (Ginther, 2005b). When multiple ovulations take place, the progesterone concentration will be higher, most likely because there are two functional CLs instead of only one. This was demonstrated in donkeys, in Portugal, by comparing the areas under the progesterone curves of single and multiple ovulations (Quaresma and Payan-Carreira, 2015).

Luteolysis is a very important phenomenon in the estrous cycle of the mare, as it will allow another cycle to begin when a pregnancy does not occur. As in other domestic species, luteolysis in the mare is first signalized by the secretion of $PGF_{2\alpha}$ by the endometrium, which is believed to be controlled by COX-2 (Boerboom, 2004). Studies in sheep (Charpigny et al., 1997) and cows (Arosh et al., 2002), had already concluded that temporary increases in the expression of COX-2 mRNA happen concurrently with luteolysis.

Oxytocin is a neuropeptide hormone produced, synthetized and kept in the hypothalamus and posterior pituitary together with neurophysin, its "carrier protein". Within its reported roles are: uterine contractility for drainage of cellular remains and uterine fluids and also contractility during parturition directly and indirectly by stimulation of the release of $PGF_{2\alpha}$; it will also interfere in late diestrus with luteolysis (Brownstein et al., 1980; Melrose and Knigge, 1989).

Oxytocin has been identified in several organs and animals, such as human placentas (Chibbar et al., 1993), rat uterus (Larcher et al., 1995), cow (Wathes and Swann, 1982; Fields et al., 1983), goat (Kiehm et al., 1989), sheep (Rodgers et al., 1983), rat (Ho and Lee, 1992), pig (Jarry et al., 1990; Nitray and Sirotkin, 1992) and baboons (Khan-Dawood, 1986). It was identified in human ovaries (Schaeffer et al., 1984; Guillou et al., 1992; Maas et al., 1992;) but not in the mares´ ovaries (Stevenson et al., 1991; Stock et al., 1995). It can be found

oxytocin in the mare's ovaries but not as neurophysin, what indicates that it is probably not secreted there (Watson et al., 1999). However, another study has identified luminal epithelium and superficial endometrial glands as oxytocin secretors in mares (Bae, 2003). The oxytocin secreted by the endometrium and $PGF_{2\alpha}$ will be part of a paracrine-autocrine system that accelerates luteolysis in mares that are not pregnant (Watson et al., 2005).

After luteolysis, both LH and FSH concentrations increased, probably due to the removal of progesterone's negative influence; it is also observable an increase in GnRH and gonadotropin pulses (Irvine et al., 2000). Considering FSH concentrations before and at ovulation, they are significantly higher 3 days after ovulation (12 ng/ml) than at the day of ovulation (Vandeplassche et al., 1981).

As for prolactin, there were noted some prolactin pulses after luteolysis followed by an increase in oestrone concentration suggesting that this substance might be involved in follicle maturation (Irvine et al., 2000).

5. Follicular waves

Before the development of the ultrasound technique, studies of follicular dynamics were done using transretal palpation. The ultrasound technique quickly proved itself to be valid and, by far, more accurate (Palmer and Driancourt, 1980; Ginther and Pierson, 1984; Pierson and Ginther, 1987). Other valuable procedures for the study of follicular waves are the distribution of follicles among size categories, study of excised ovaries, ovariectomies and steroid treatments with the consequent emergence of a new wave (Driancourt et al., 1982a, 1982b; Ginther, 1993).

Besides the ultrasonographic day-to-day studies (Bergfelt and Ginther, 1992), other experiments were conducted without maintaining track of each follicle identity, using mathematical methods and reaching similar conclusions (Donadeu and Pedersen, 2008; Ginther and Bergfelt, 1992). Mathematical methods have some advantages – require less skilled operators, data collection and interpretation is easier and quicker, and the emergence of a follicular wave can be detected earlier (Ginther and Bergfelt, 1992). Concrete data demonstrate that by the identity method, divergence was occurring 2 days before its detection,

when follicles were about 15 to 16 mm. With the mathematical approach, divergence was happening only 5 days before its first detection, when follicles were about 9.5 mm (Ginther and Bergfelt, 1992). An apparent limitation of the statistical method might be related with the overlapping of simultaneous waves (Ginther, 1993). Another limitation is the difficulty in differentiating healthy and growing follicles from the ones undergoing atresia (Pierson and Ginther, 1987).

Initial studies, started to recognize that follicles developed in cohorts more or less synchronized and deeply influenced by gonadotropin concentrations. It was even suggested that there were two waves of follicular growth per cycle, being the last one, the one that would originate the ovulatory follicle (Irvine, 1981). With the evolution in techniques and the continuous study of this subject, other conclusions were taken and currently it is accepted, in mares, that the more common wave pattern is the one wave model (Sirois et al., 1989; Ginther, 1992). However, 1 to 2 follicular waves are expected during the estrous cycle that follows the transitional periods and are also normal to happen during the breeding season (Bergfelt and Ginther, 1992). Differently from heifers, to which the expected pattern is 3 waves per cycle with a mean interval of 7 days between various wave emergence and starting on the second day after ovulation (Sirois and Fortune, 1988). Primates are another example of species who display waves of follicular activity (diZerega et al., 1980; Hodgen et al., 1985).

Differently from cows, in mares and women not all the follicular waves detected will result in the selection of a dominant follicle, leading to the definitions of major *versus* minor waves (Mihm and Evans, 2008). A major follicular wave is defined by a cohort of follicles that starts to develop together, but will eventually diverge into dominant and subordinate follicles. These waves can also be classified as primary waves, if they emerge in the second half of the cycle and the dominant follicle manages to reach ovulation and ovulates, or secondary waves if it emerges in the first half of the cycle and the dominant follicle ovulates or regresses, ovulates during diestrus or becomes a hemorrhagic follicle (Ginther, 1993). The presence of large follicles during diestrus, that sometimes ovulate, is a sign of the presence of these secondary waves besides the primary ovulatory wave (Sirois et al., 1989; Ginther, 1990; Donadeu and Pedersen, 2008). The occurrence of diestrus ovulations varies among equids (Wesson and Ginther, 1981). Mares, in these cases, generally do not show estrus signs due to the effect of progesterone. They might instead present a prolonged luteal phase (Stabenfeldt et al., 1974;

Douglas and Ginther, 1975; Hedberg et al., 2006). In minor waves, the largest follicle, for some reason, fails to accomplish dominance and will eventually regress together with the remaining follicles of the wave (Ginther, 1993). During both major and minor wave emergences there is a significant increase in mean diameters of the largest subordinate follicles and of the largest follicle, respectively (Bergfelt and Ginther, 1992).

A predeviation follicle is described as being part of the ovulatory wave and having a typical large size that would make him a candidate to become the dominant follicle, but still, fails to undergo deviation and regresses. This might take place due to an asynchrony between the follicle that has already grasped a deviation size and the mechanism of deviation that is not yet ready to occur. Double dominant follicles are follicles that exceeded both the maximum diameter at the beginning of deviation and might both ovulate or not. In these cases, the third larger follicle is considered to be the largest subordinate follicle. A major anovulatory wave is known for comprising a dominant follicle that will fail to ovulate (Ginther et al., 2004b).

Results of different follicular dynamics studies show the existence of primary waves, which emerged between day 3 and 14 of the estrous cycle, and secondary waves, that emerged at day 1.4 ± 1.6 before ovulation. Longer interovulatory intervals were found to be positively correlated with late emerging primary waves. Secondary waves generally developed during diestrus and either ovulate or suffer atresia. Largest follicles of secondary waves show a growth rate during the first 8 days after wave emergence similar to what is observed in primary waves. However, they have a tendency to diverge earlier and not to reach as large proportions as the dominant follicle of a primary wave does. Secondary waves and primary waves can both be major waves (Bergfelt and Ginther, 1992; Ginther and Bergfelt, 1992; Ginther, 1993).

Some minor waves were found at the studies presented above. The authors hypothesized that even more waves could have been found if not for the method of detection used. Using larger follicles excludes all the smaller follicles that eventually are part of a developing minor wave. When compared to primary waves, minor waves have smaller sizes of its largest and smallest follicles, but no significant differences for follicular diameters at emergence. Comparing to secondary waves, minor waves present different maximum follicular diameters. Minor waves, as expected, did not present follicles reaching divergence and emerged either before the

emergence of the primary wave or 2 to 7 days before ovulation (Bergfelt and Ginther, 1992; Ginther, 1993). It was observed by Ginther (1993) a higher occurrence rate of minor and secondary waves throughout spring cycles, partly justifying the apparent bigger follicular activity at this season of the year.

Multiple ovulations may originate from a primary wave or from the primary and secondary wave. Actually, Ginther (1993) observed three different situations. Mares that ovulated two follicles from the same primary wave, mares that had one ovulation originated from the primary wave and another one from a secondary wave. The third situation entailed an ovulation from the current primary wave and another one from the primary wave of the previous cycle. Similar results were reported before (Bergfelt and Ginther, 1992).

These studies pointed out some of its own limitations. For example, when successive estrous cycles have a dominant follicle from the same ovary, there is a period of time at the beginning of the cycle during which information is lost. What happens is an overlapping of information from the regressing wave and the new emerging wave to which the authors call "area of ambiguity" (Ginther and Bergfelt, 1992). The interpretation of this information tends to become somewhat arbitrary. Cavilla et al. (2013) have demonstrated that the overlapping of follicular waves is a very typical pattern, detected in lammas, possibly due to genetics, but also by nutritional and environmental factors.

Another phenomenon observed is that large ranges in the diameters of large follicles might also overlap information and make it more difficult to find significant waves of follicular activity. This occurs because regressing subordinate follicles are not always fully regressed at the time of emergence of the new primary wave. The solution presented was to remove the follicles with larger diameters from the graphic results. The overlapping of follicles from two distinct waves was also described in women, though apparently in a lower rate (Ginther et al., 2004b).

The frequency of FSH surges, the number of surges per mare and the peak concentrations of this hormone is significantly higher before the emergence of follicular waves. Mean FSH concentrations increase 6 to 8 days prior emergence, to only decrease 1 day before to 2 days after emergence of the primary follicular wave. FSH concentrations are also found to increase 2 days before to 1 day after the previous ovulation in mares with minor follicular waves.

Moreover, FSH concentrations are higher between day 1 and 3 in mares with minor follicular waves. After day 3, FSH concentrations increase when no minor wave occurs and remain unaltered when there is a minor wave (Bergfelt and Ginther, 1992).

6. Anovulatory follicles

In mares, persistent anovulatory follicles are likely to occur in 11.9% of the cycles during the breeding season and in 22.2% of the cycles during the transitional phase with a peak of occurrences in November (Paccamonti, 2012). Usually, the follicle has an apparent normal development until it reaches a mean of 48.1 ± 8.0 mm in diameter (Lefranc et al., 2003). More recently, Ginther et al. (2007b) have reached similar conclusions and moreover, have noted an apparent repeatability among individuals and anovulatory hemorrhagic follicles to be more common in older mares.

Contrary to these results, in 2009, a study observed no significant differences in the prevalence of hemorrhagic follicles between seasons. The same study reports a higher frequency of hemorrhagic follicles associated with the administration of Cloprostenol and hCG (Cuervo-Arango et al., 2009). About the etiology of these structures, besides Cloprostenol and hCG, studies testing the effect of Flunixin Meglumine were done. It was concluded that this drug can induce persistent anovulatory follicles that will luteinize and produce progesterone (Cuervo-Arango et al., 2011).

Prediction of the occurrence of persistent anovulatory follicles is not easy. Authors have reported no significant differences in uterine edema between an ovulatory and anovulatory cycle (Cuervo-Arango and Newcombe, 2009). In B-mode ultrasonography, the hemorrhagic follicles' wall becomes thicker and the antrum loses its anechogenicity as luteal tissue develops (Lefranc et al., 2003). Using Doppler ultrasound, a more developed vascularity was observed in the apical area in future hemorrhagic follicles (Ginther et al., 2007b; Paccamonti, 2012).

There are studies that present conflicting results in what concerns to hormone profiles when ovulation fails to occur. Some authors report progesterone concentrations above 1 ng/mL (Lefranc et al., 2003; Ellenberger et al., 2009; Cuervo-Arango, 2011), while others have not

found any substantial differences among progesterone, estradiol and LH concentrations between females that ovulated and the ones that failed to achieve ovulation (Ginther et al., 2007b). It was reported that waiting for this structure to regress naturally might be better than using hormonal treatments (Paccamonti, 2012). The use of substances such as $PGF_{2\alpha}$ analogues, that will induce luteolysis, was associated with a higher occurrence of hemorrhagic follicles (Lefranc et al., 2003).

7. Seasonality

7.1. Mares

Ovarian dynamics and both reproductive systemic and local regulatory mechanisms during the non-breeding season have been far more studied in horses. Photoperiod, nutrition and body condition, temperature and age are the most discussed factors that can influence reproductive activity. However, recent studies have demonstrated other mechanisms by which the reproductive activity can be modulated (Nagy et al., 2000). It has been demonstrated that there are ovarian mechanisms that regulate reproductive activity besides hypothalamus and pineal glands activity (Donadeu and Watson, 2007). Horses are long day breeders and display a marked follicular activity suppression during winter months (Snyder et al., 1979). The anovulatory period reported for mares can be subdivided in two transitional periods, fall or vernal and spring transitions, and a deep anestrus period situated between the two transitional periods already mentioned (Ginther, 1990; Donadeu and Watson, 2007).

A decrease in follicular diameters during fall transition period occurs (Snyder et al., 1979; Ginther et al., 2003b) and a higher rate of anovulatory follicles during both transitional periods and deep anoestrus is observable (Nequin et al., 2000; Donadeu and Ginther, 2002b; Watson et al., 2002a). During the non-breeding season, a drop in cell proliferation from antral and pre-antral follicles leads to a low ovarian activity level (Driancourt et al., 1983; Donadeu and Ginther, 2002b). Before normal follicular activity returns, a spring transition period is expected, where a slow or sudden increase in follicular activity ensues. (Freedman et al., 1979; Snyder et al., 1979; Carnevale et al., 1997; Donadeu and Ginther, 2002b).

Before the first ovulation of the year, Ginther (1990) observed 8 out of 14 mares developing large dominant follicles during the spring transitional period. No apparent pattern of follicular

growth and regression was detected before this period (and until ovulation within the remaining 6 mares). However, it had been suggested before a wave-like pattern for the anovulatory season in mares (Sirois et al., 1989) later confirmed (Nequin et al., 2000; Donadeu and Ginther, 2002b; Donadeu and Pedersen, 2008) and characterized by the absence of deviation during deep anoestrus (minor waves) and the presence of dominant follicles during spring and fall transitions (Ginther et al., 2001a, 2004a; Watson et al., 2002b; Ginther et al., 2003a; Donadeu and Ginther, 2003a, 2003b, 2004).

7.2. Jennies

Donkeys present less marked seasonal effects than horses (Ginther et al., 1987). Nevertheless, different reports describe the jennies' reproductive activity differently, which can be a reflection of the application of different methods, different breeds or different environmental conditions among other aspects (Donadeu and Watson, 2007).

In 1986, 12 jennies were housed in Wisconsin (USA) and it was observed that 50% of the jennets kept their normal reproductive activity, ovulating year-round. Among the remaining 6 jennies, there were 2 that presented a persistent CL that caused an anovulatory period. It was also observed, in 4 jennies, what seemed to be a demonstration of seasonal effects. These jennies presented a prolonged interovulatory interval, from November to January. This period was characterized by a luteal phase of about 14 days and an estrus phase of 17 to 41 days. It was noticeable the ovulatory and anovulatory seasons, with the last one happening during the winter months, lasting only 39 to 72 days followed by the prolonged period of follicular activity and estrus behavior mentioned above (Ginther, 1986).

Ginther et al. (1987) observed seasonal effects in the length of interovulatory intervals and estrus periods, with both lasting longer during winter months. A higher occurrence rate of seasonal anestrus has been reported, with six out of thirteen jennies presenting an anovulatory season with irregular estrus periods and only 2 mares cycling regularly throughout the whole experiment. Diestrus was not apparently affected by season while estrus period tended to be longer at the beginning and end of the breeding season (Henry et al., 1987).

Different results were reported more recently for the *Martina Franca* breed including an increased estrus and decreased diestrus duration during summer months. All the jennies included in the study presented normal estrous cycles and behavior during the entire year (Contri et al., 2014). Previously, reduced seasonal effects were already reported for *Martina Franca* jackasses (Carluccio et al., 2013), with Contri et al. (2014) suggesting a possibly different seasonal effect on the hypothalamic-pituitary-gonadal axis than the one observed in horses. Also reported were increased follicular diameters during spring and summer months. Estrogen and progesterone profiles presented no seasonal effects (Contri et al., 2014).

The reproductive patterns during the non-breeding season were also studied for the *Miranda* donkey breed (Quaresma et al., 2015), with the observation of jennies cycling normally year-round and also cases of disruption of normal cyclicity during winter months. Of a total of 12 jennies, 5 went through an anestrus period, 3 presented silent estruses and 1 had a prolonged CL.

7.3. Endocrine regulation during the anovulatory season

In regard to the endocrine regulation during the non-breeding season, evidence show that follicular waves development is preceded by FSH surges, as observed during the breeding season. However, the extent of FSH surges does not seem to convoy the tendency for bigger and smaller follicles during spring and fall transitions, respectively. Neither do they seem to be related with low follicular development levels during deep anestrus (Donadeu and Ginther, 2002b; Ginther et al., 2003a). On the other hand, the considerable variety of FSH isoforms still leaves the necessity of studying if, as in humans, rats and sheep, the production of different isoforms in the mare is influenced by season or if the expression of FSH receptors might change throughout the year (Moore et al., 2000; Donadeu and Watson, 2007).

LH concentration in the follicular fluid was measured in dominant anovulatory and ovulatory follicles together with estradiol, IGF-I, inhibin-A and VEGF concentrations. Anovulatory follicles presented lower concentrations of these substances and also less vascularity (Ginther et al., 2003b; Acosta, 2004b). During non-breeding season, LH surges cessation is caused by a shutdown in pituitary LH synthesis and the reduced follicular diameters. The absence of

ovulations will last as long as the low LH surges do, revealing the considerable importance this hormone has for the growth of dominant follicles (Snyder et al., 1979; Silvia et al., 1986, 1987; Nequin et al., 2000; Bergfelt et al., 2001; Ginther et al., 2003a; Donadeu and Ginther, 2003a, 2003b, 2004).

Dopamine is a catecholamine that increases its concentration during the anovulatory season (Melrose et al., 1990). Dopamine appears to be associated with follicular growth in mares. The relation and dynamics between dopamine and FSH and dopamine and prolactin might affect follicullar development (Donadeu and Watson, 2007). Although not effective during deep anestrus, dopamine antagonists were discovered to increase follicular activity during the time of spring transition. Also, dopamine agonists were found to have the opposite effect, inhibiting follicular activity during the same period of time (Besognet et al., 1997; Daels et al., 2000; Nagy et al., 2000; Donadeu and Thompson, 2002). More recent study results indicate a temporal relationship between increase in dopamine receptor D2 and decrease in FSH concentrations; whether there is a cause-effect relationship it is not sure (King et al., 2008). Dopamine is likely to affect directly the ovaries. In fact, it was reported, that the ovaries of mares present 2 types of receptors for this substance (King et al., 2005).

The interaction between dopamine and prolactin is likely to affect reproductive activity during anovulatory season. Dopamine might influence the secretion of prolactin by a negative feedback mechanism (Nagy et al., 2000; Donadeu and Watson, 2007; King et al., 2008). Prolactin is a pituitary hormone that increases during spring and decreases during fall and winter (Johnson, 1986). According to Melrose et al. (1990), increases of prolactin concentrations might occur as a consequence of the decrease in dopamine concentration. With a role in physiological changes like fur shedding, prolactin might also have a role in seasonal reproduction as it was studied in other species and also in the mare (Fitzgerald and McManus, 2000). In the mare, the administration of porcine prolactin seems to be effective in promoting ovulation and luteal tissue formation (Saito and Saxena, 1975; Thompson et al., 1997 ; Donadeu and Thompson, 2002; King et al., 2008).

Follicular steroids, as progesterone and estradiol, usually decrease in concentration alongside with fall transition and deep anestrus, affecting follicular diameters. During spring transition, follicles can manage to reach large diameters again, even with steroid levels still rising and

only reaching regular values near the onset of the breeding season (Davis and Sharp, 1991; Watson et al., 2002b; Ginther et al., 2003a). In 2004, a study concluded that large transitional follicles present limited ability to synthetize androgens, estrogens and progesterone when compared to preovulatory follicles during the breeding season. They also evidenced low levels of vascularization, that combined with the low steroidogenic capacity will stop the follicle from reaching adequate LH levels and as a consequence, ovulation during non-breeding season will not happen (Watson et al., 2004a).

Higher levels of IGF-I were found to be present in mares that presented a higher reproductive activity during winter season. IGF-I concentration was also found to be lower during spring transition. However, a posterior study concluded that IGF-I levels are determined systemically and differences in its levels did not interfere with follicular development during spring transition period (Gentry et al., 2002b; Acosta, 2004b; Watson et al., 2004b). The above mentioned results leave many doubts about the role of IGF system during the anovulatory season. (Donadeu, 2006) proposed that IGF system might be involved together with other trophic factors, such as inhibin-B, instead of having a preponderant role by itself.

VEGF has been found to be present in higher concentrations in dominant follicles rather than in subordinate follicles, promoting a better vascularization and this way a better trophic support for its development (Acosta, 2004a). When comparing same sized follicles, Doppler ultrasonography revealed that the ones from transitional periods present poorer vascularity than the ones from the ovulatory season; other tests pointed a less developed internal theca and granulosa layer, lower level of factor VIII and a lower concentration of VEGF (Watson and Al-Zi'abi, 2002a; Acosta, 2004b). Low levels of LH and IGF-I are believed to be related with low angiogenic factors such as VEGF, poor vascularization and vascular permeability and early follicular atresia (Watson and Al-Zi'abi, 2002b; Ginther, 2003b; Acosta, 2004b).

During deep anestrus, small follicles still manage to produce inhibin. However, concentration of total inhibin decreases during transitional period into the deep anestrus period (Irvine et al., 2000). Following the same tendency as other substances mentioned above, during spring transition inhibin concentration will rise progressively (Donadeu and Ginther, 2003a; Watson et al., 2002a).

7.4. Other factors that interfere with seasonality and the estrous cycle

7.4.1. Photoperiod

Early studies from the 70s already proposed that temperature and photoperiod were preponderant factors for the onset of the breeding season (Davis and Meyer, 1973; Kooistra and Ginther, 1975; Sharp and Ginther, 1975; Garcia and Ginther, 1976; Freedman et al., 1979). Both Davis and Meyer (1973) and Garcia and Ginther (1976) proposed the coexistence of two mechanisms for the regulation of seasonal reproductive activity. The first one was believed to be altered by an environmental signal, most likely the photoperiod, which should stimulate the hypothalamic-pituitary axis. The substance secreted by this axis would then stimulate the secretion of pituitary gonadotropins that will stimulate the ovarian activity. Only at this point could the second mechanism start, including negative feedback mechanisms of endogenous steroid hormones and external behavioral interactions. Later, changes in melatonin secretion by the pineal gland were found to alter GnRH (and gonadotropins) secretion during fall and winter.

During winter photoperiod, more night hours lead to long melatonin secretion periods and to a reduced level of follicular activity and anestrus. A long night can be defined as a biological rhythm in which melatonin is present 10 hours after nightfall (Ginther, 1992; Palmer and Guillaume, 1992; Donadeu and Watson, 2007). Melatonin was undeniably associated with seasonal changes in reproductive activity when studies that contemplated administration of exogenous melatonin or pinealectomies changed the effect of daylight and reproductive alterations were observed (Grubaugh et al., 1982; Palmer and Guillaume, 1992). An optimal stimulation of reproductive activity using photoperiod is believed to include 14.5 to 16 hours of light per day. Also, it was reported that long continuous periods of day light might have the opposite effect of the one desired, as mares may become refractory to the day light effect (Oxender et al., 1977; Palmer and Guillaume, 1992).

When discussing the effect of photoperiod and temperature on the onset of the breeding season, Sharp and Ginther (1975) and Palmer and Guillaume (1992) questioned whether it was the intensity of the stimuli or the duration in time and rhythm of the stimuli that was crucial for an effective induction of reproductive activity. These authors presented positive

results on inducing return into cyclic activity. However, they were not able to separate the effect of temperature from photoperiod due to the design of their study.

Even though photoperiod might be the most important external factor modulating reproductive activity in mares, during winter not all animals exposed to the exact same photoperiodic conditions express equal responses which can be explained by the influence of other factors other than photoperiod (Nagy et al., 2000).

7.4.2. Nutrition and body condition

It was already mentioned how jennies do not present as patent seasonality characteristics as mares (Ginther et al., 1987; Aurich, 2011), attributed possibly to a different effect of photoperiod on the hypothalamus, pineal gland and gonads (Contri et al., 2014) besides the climate and latitude (Quaresma et al., 2015). Studies on seasonality and reproductive activity in the jackass suggest a similar premise (Carluccio et al., 2013).

A study of the reproductive system of tropical jennies reported a different effect of photoperiod on these donkeys. These jennies presented a lower ovulatory rate during the dry season when there are more light hours per day. The forage availability during the rainy season and their body condition improvement is the justification presented by the authors for this reproductive behavior (Lemma et al., 2006b).

Body condition and nutrient intake are major factors that can influence animals in all different reproductive states (Henneke et al., 1983). Fitzgerald and McManus (2000) proposed that besides photoperiod, the amount of body fat and the quantity of leptin (a hormone produced by adipocytes) influenced GnRH and the gonadotropins release, and therefore mares' seasonality. Mares in low body condition usually present low leptin concentrations and are likely to present a much more pronounced anestrus period during winter (Gentry et al., 2002a). Similar findings were reported in *Lusitano* mares (Ferreira-Dias et al., 2005) and in Brazilian *Campolina* mares (Zúccari et al., 2013). Reversely, high body condition scores, together with high insulin and leptin levels, are believed to not alter reproductive characteristics during the non-breeding season (Waller et al., 2006). Also, mares with low body condition presented higher number of major anovulatory waves and smaller

preovulatory follicles, than the ones that had an adequate body condition (Davies Morel et al., 2010).

In beef cows, a high nutrient intake was reported to shorten the interval from parturition until estrus and pregnancy rate at first estrus (Ciccioli et al., 2003). In mares, different authors have reached similar conclusions on the effect of nutritional status and amount of body fat on the occurrence and severity of seasonal anestrus and low ovarian activity (Henneke et al., 1983; Gentry et al., 2002a, 2002b). In donkeys, Quaresma et al. (2015) found BCS to influence ovarian activity in *Miranda* donkey breed jennies during the non-breeding season. Jennies with poor body condition presented lower ovarian activity, and only when an increase in their body condition was observed would they return to normal reproductive activity. Similar results were found in free ranging tropical jennies. Higher follicular activity was seen between March and September. Improvements in feed type, nutritional status and changes in management were associated with better follicular development during transitional periods, in opposition to the dry season when reproductive performance was low (Lemma et al., 2006a).

7.4.3. Age and parity

In mares, there is an age effect in the development of follicles larger than 11 mm. Mares from 20 to 26 years old present longer interovulatory intervals, caused by an extended follicular phase and low LH concentrations. There is a registered decrease in follicles' diameter and a decrease in their growth rate. Older mares also tend to present alterations at the ovulation fossa. During transitional periods it was observed that younger mares presented greater follicular activity than older ones (Carnevale et al., 1997). Actually, older mares are less reproductively competent; the main reasons are loss of uterine health and decrease in oocyte viability (Davies Morel et al., 2010). Conversely to the results obtained for horse mares, a recent study with *Miranda* donkey breed jennies observed more exuberant seasonal effects among younger jennies. The authors proposed the lower body condition of these younger donkey mares (that present increased metabolic imbalance) as an explanation for these findings (Quaresma et al., 2015).

Pregnancy and the number of pregnancies a mare went through might cause alterations to follicular dynamics according to the stage of pregnancy. Follicular dynamics during the first half of gestation are compared to what is observed during the first half of the anovulatory season with sporadic major waves or only minor waves (Donadeu and Pedersen, 2008). This decrease in follicular activity might be caused by progesterone negative feedback on LH concentrations (Ginther et al., 2003a). After parturition a general positive effect is observed with an increase in follicular diameters and activity (foal heat). These effects might vary within each mare and with the foal's presence or not (Ginther, 1992; Nagy et al., 1998).

7.4.4. Presence of the stallion

The stallion, also used for estrus detection during the breeding season (Vandeplassche et al., 1981; Contri et al., 2014; Quaresma and Payan-Carreira, 2015), can be used to aid the return to cyclicity after the non-breeding season. In mares, the stallion's presence shortens the time to the first ovulation of the season and increases the ovulation rate (Wespi et al., 2014). Similar results were obtained in sheep and goats, probably due to the presence of pheromones, male specific odors or vocalizations (Claus et al., 1990; O'Callaghan et al., 1994; Gelez and Fabre-Nys, 2004; Janson and McDonnell, 2010).

8. Ultrasonography and ultrasonographic aspects of ovarian structures

Transretal ultrasound is a very useful technique for reproductive examination in horses (Ginther and Bergfelt, 1992), cattle (Ginther et al., 2001b), donkeys (Quaresma and Payan-Carreira, 2015), llamas (Cavilla et al., 2013) and mules (Volpe et al., 2005). It is used for visualization of the uterus and ovaries: to evaluate ovarian activity, to estimate the current estrous cycle stage, to assess follicular dynamics and possible ovulation failures and to check pregnancies, detect and prevent double ovulations and detect silent estruses. Although it is a very useful technique, care must be taken so that reliable results can be obtained. Rotation or change in position of an ovary or presence of two dominant follicles at the same ovary can lead to failure in detecting certain structures, also different operators and different levels of experience might lead to different results (Miro, 2012).

In equids, ovaries present a homogenous and moderately echogenic ultrasound image (Miro, 2012) with spherical anechoic structures filed with fluid that correspond to ovarian follicles and that can achieve considerable sizes (Ginther and Pierson, 1984) (Figure 4). *Corpus luteum* is a brighter (more echogenic) ovarian structure than follicles, with a pear-like shape (Kimura et al., 2005) (Figure 5). It can have a variable central cavity that can be anechoic in case it's filled with blood or echogenic if filled with fibrin-like material. These cavities are susceptible to being mistaken for follicles (Ginther and Pierson, 1984). Jennies present echogenic corpus luteum with the fibrous centrum more echogenic than the peripheral luteal tissue. Color Doppler, as for other structures, is the best technique to evaluate the CL's functionality (Taberner et al., 2008; Miró et al., 2015).

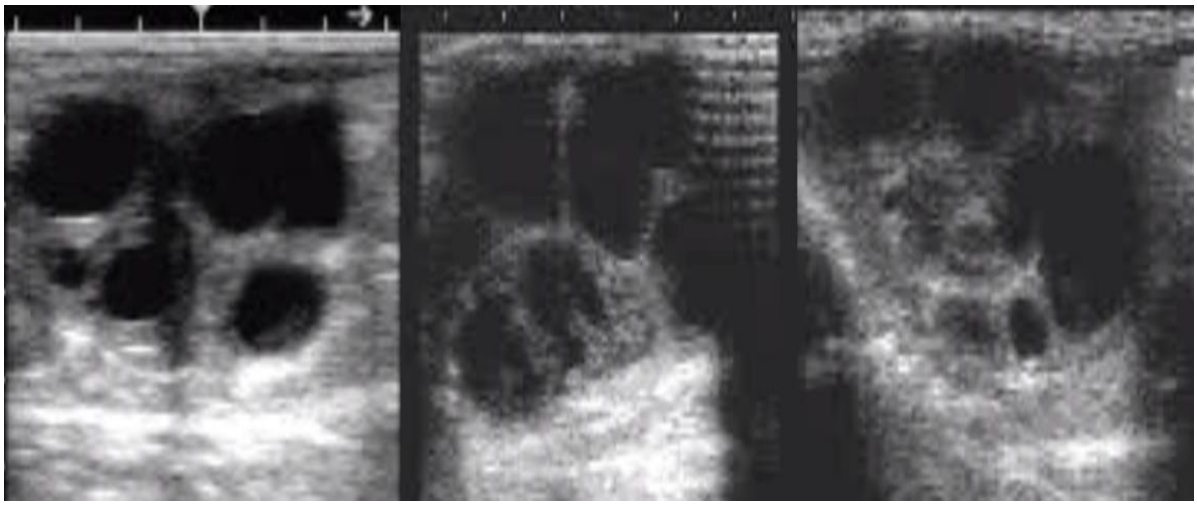

Figure 4 Ovarian ultrasonographic image, showing multiple different sized, anechoic follicles *(Photo courtesy of Miguel Quaresma)*

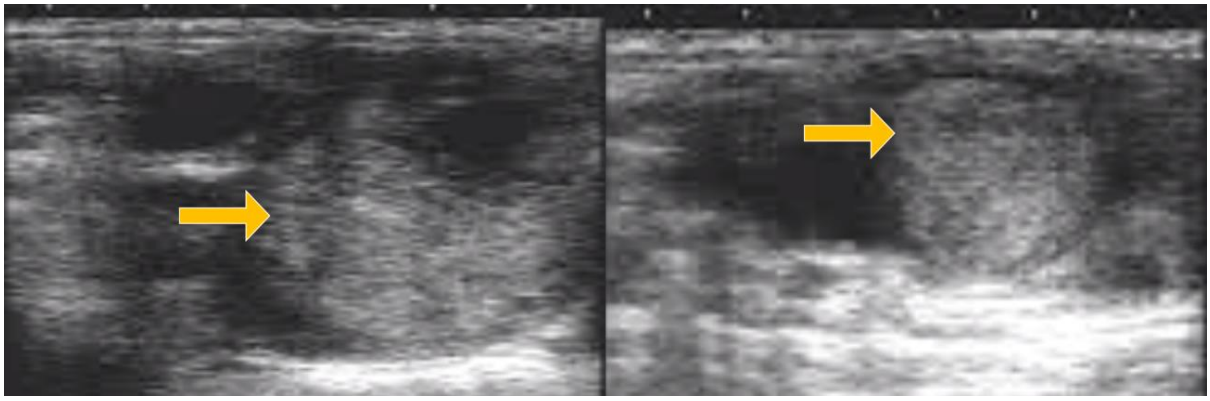

Figure 5 Ultrasonographic view of an ovary. The arrows show two corpus luteum *(Photo courtesy of Miguel Quaresma)*

When ovulation fails to occur, hemorrhagic follicles are present at the ovary. Their ultrasonography image is characterized by a granular appearance with an echogenic fibrous septum (Miro, 2012). Ovulation occurs exclusively at the ovulation fossa in the mare, and can be detected by the sudden disappearance of the large dominant follicle on the ovulation day and the later appearance of a corpus luteum (Ginther and Pierson, 1984; Kimura et al., 2005). It is easier to detect the moment of ovulation when ultrasound exams are performed within

short intervals of 4 to 6 hours (Figure 6). On the 24 hours preceding ovulation, usually there is a cessation of growth of the dominant follicle or even a decrease in its diameter and a shift to a more conical shape that can be detected with a reproductive ultrasound exam (Palmer & Driancourt, 1980; Ginther, 1986). Also, the granulosa layer becomes more serrated and external and internal theca – follicular wall becomes thicker. Vascularization will develop before ovulation and can be detected by Doppler ultrasonography at the external theca (El et al., 1999; Shirazi et al., 2004; Palmer, 2006; Miro, 2012).

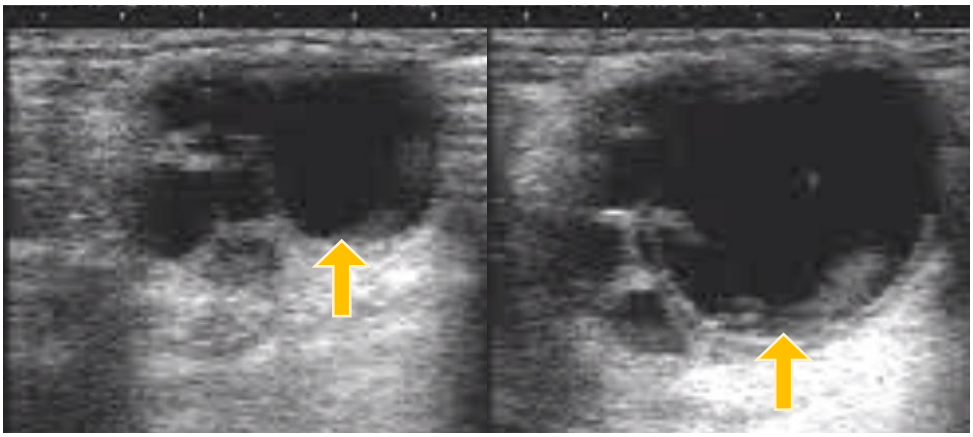

Figure 6 Images from the left ovary of a *Miranda* jenny at the onset of estrus (left) and close to ovulation (right). The dominant (left) and pre-ovulatory follicle (right) are indicated with a yellow arrow. It is possible to notice the change in size and shape of the dominant follicle until ovulation. It is also observable the presence of subordinate follicles at the left side of the dominant follicle *(Photo courtesy of Miguel Quaresma)*

The non-breeding season can also be monitored by ultrasound technique. It is expectable to detect low ovarian activity with a low number of follicles developing and no corpus luteum are present. The ovarian parenchyma looks homogeneous; the uterus presents no edema and also has a homogenous look (Figure 7).

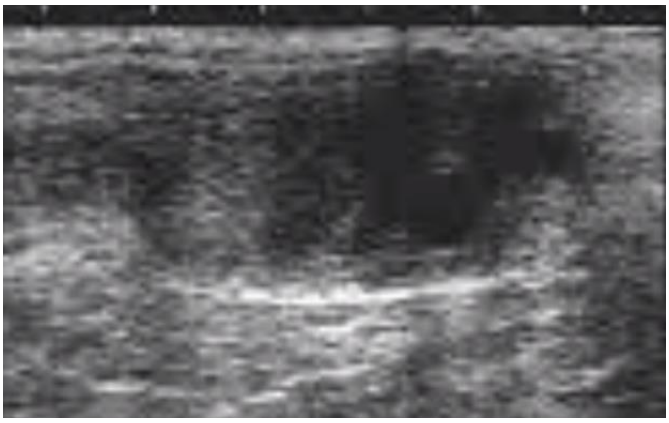

Figure 7 Ultrasonographic image of an ovary of a *Miranda* jenny during the seasonal anestrus (December) with few follicular structures and of small sizes *(Photo courtesy of Miguel Quaresma)*

During transitional periods there are expected many follicular waves that develop and then regress. The uterus might present an edematous image. However, it has been mentioned that the uterine edema is not a reliable indicator of ovulation (Watson et al., 2003). Doppler ultrasonography is useful to distinguish dominant follicles that might ovulate from the ones that will regress (Miro, 2012).

II. Objectives

The main goals of this study were to:

a) Measure and register diameters of all the follicles developing in the ovaries of 15 *Miranda* jennies during the non-breeding season;

b) Characterize the observed follicular wave patterns during the non-breeding season in *Miranda* jennies;

c) Study and describe the factors affecting follicular wave patterns in these jennies breed;

d) Contribute to new information to the reproduction study of the *Miranda* donkey breed in particular and to donkey reproduction in general;

e) Understand the estrous cycle of the donkey species;

f) Help to prevent continuous decline of European donkey populations, as well as to open paths to year-round foaling, if deemed necessary.

III. Materials and methods

1. Animals and management

This study used 15 non-pregnant females of the *Miranda* donkey breed, aged 3 to 18 years old. All jennies were healthy and their BCS ranged from 4 to 7 (on a nine-point scale). After a breeding soundness exam, all jennies were considered potentially fertile and all were cycling at the beginning of the observations. Of the fifteen jennies, jenny 1 was excluded due to insufficient data collection.

Data was collected from the beginning of September until the end of April, including periods of vernal and spring transitions, during two consecutive years, with similar climate conditions (Quaresma et al., 2015). The period between October 31st and March 30th was considered for statistical analysis on the occurrence of different types of follicular waves.

The seasonal periods definition was based on how reproductive seasons are defined by different authors (Blanchard and Blanchard, 2003; McKinnon, 201; Atayde and Rocha, 2011).

The animals were housed at the University of Trás-os-Montes e Alto Douro in Vila Real, Portugal (41º17'N 7º44'W) in a 2500 m^2 paddock under natural photoperiod conditions. The animals had a 50 m^2 shelter granting protection from the sun, wind and rain. Each jenny was fed twice every day with: 5 to 7 kg of hay and straw and 400 g of concentrate. Clean fresh water was always available. All jennies were vaccinated for Equine influenza and tetanus (ProteqFlu-Te, Merial S.A.S., Lyon, France) and dewormed twice a year with 200 µg of ivermectin per kg of body weight (Noromectin Oral Paste, Norbrook Laboratories, Northamptonshire, UK). All animals were handled in accordance with EU Directive 2010/63/EU for animal experiments.

Jennies were teased daily with a jack and were considered in estrus if demonstrating the behavioral estrus sign of mouth clapping together with at least another one of the behavioral signs: winking and urinating, tail raised or posturing. Jennies were considered to be non-receptive when presenting their tail down, lacking interest or presenting an aggressive behavior towards the jackass.

Sequential US records were used to establish the moment of ovulation as the middle point in time between two US scans when a dominant follicle ceased to be observed during estrus. The beginning of estrus was set at the moment when the female first showed signs of receptivity to the male. Diestrus corresponded to the period when the female refused the jack, with the presence of at least one active *corpus luteum*. They were considered to be anestrus when the female refused the jack, without the presence of a large pre-ovulatory follicle or signs of behavioral estrus, as well as in the absence of an active *corpus luteum.* The ovulation rate was defined as the number of ovulated follicles, on the basis of US observation of the collapse of the preovulatory follicle or follicles.

A follicular wave was considered to be any apparently organized follicular dynamics where a group of follicles start to grow and regress. A minor secondary wave was considered to be present when a group of follicles starts to grow together, reaching a maximum diameter and then regresses without any of them reaching dominance. A major secondary wave was considered to be any wave that emerged in the first half of the cycle and presented a general tendency of its follicles to grow initially at a similar pace but with some reaching divergence and others becoming subordinates and regressing, during progestagenic phase. Dominant follicles of this type of waves either ovulated or failed to ovulate.

The end of a wave was defined by the maximum size of the largest follicle. It was not possible to assert the beginning of this waves because growing follicles and regressing follicles from a previous wave can be found at the same time in the ovaries. Primary or major ovulatory waves include an initial phase where follicles grow at a similar pace followed by divergence where dominant and subordinate follicles become evident and that ends at the moment of ovulation.

2. Assessment of follicular sizes

Routinely ultrasound exams were done every other day during diestrus and every 12 hours during estrus periods. A linear array US scanner with a 5 MHz linear transretal transducer (Shenzhen Veterinary US Scanner) were used for every examination,

coupled to a video camera (DCR – HC96E, Sony) that recorded the examinations. As ultrasounds were mostly done every other day, the identity of each follicle was not kept.

To obtain follicular diameters, follicular antrum were measured and an average of its narrowest and widest dimensions was measured using ImageJ® software (https://imagej.nih.gov/ij/index.html) on fixed frame images. All measurements were done by the same operator, though a different operator from the one who did the ultrasound examinations.

Dominant follicles were considered to be those that continued to grow after deviation while the other subordinate follicles started to regress. Ovulatory follicles were defined as a dominant follicle that reached ovulation, while the ones that failed to ovulate were called anovulatory follicles. Day of ovulation was set as day 0. Interovulatory intervals were defined as the period or number of days between ovulations of successive cycles or between the last ovulations in case of multiple ovulation cycles. Ovulation was ultrasonically detected by the absence of the dominant follicle from one examination to the other.

3. Statistical analysis

Data was organized using Microsoft Excel 2013 for Windows (Microsoft, USA). All US exams with less than 5 diameters measured were excluded from the study. The three largest follicles of each ovary were classified as the largest follicles and the remaining as the smallest follicles. Of the three largest follicles, and after deviation, the follicle that reached dominance was classified as the dominant follicle.

Statistical analysis was performed using JMP software from SAS Institute Inc. for Windows (Microsoft, USA). A total of 15,159 follicles were measured. Each cycle was analyzed individually, taking in consideration all follicles present in both ovaries. Using the JMP "Fit Y by X" tool, graphs were done where all follicles, measured during each ultrasound examination, appear organized by size.

Blank spaces correspond either to periods of time where no ultrasounds were done, due to logistical reasons, or to days excluded for having less than five follicles measured. Data is presented by mean ± standard deviation.

IV. Results

1. Ovarian patterns during de non-reproductive season

Five of the fourteen studied jennies kept cycling normally throughout the entire study. These animals (jennies 6, 7, 8, 9 and 13) retained a normal ovarian activity, with estrous behavior, during the non-breeding season. The remaining 9 jennies suffered some sort of disruption in their normal cyclic activity, but seasonal effects were different among these females. Jennies 2, 10 and 14 presented a variable number of silent estruses, while jenny 15 had a persistent CL and jennies 3, 4, 5, 11 and 12 went through a period of seasonal anestrus. Considering the estrous cycles recorded on all groups, mean estrous cycle length for the whole studied population (n=14) was 24.69 ± 3.95 days. Overall, estrus lasted 7.18 ± 3.68 days, ranging from 23 to 1 day. Diestrus periods lasted 18.03 ± 4.30 days ranging from 36 to 6 days. The results presented above do not comprehend data from one cycle of jenny 15 that comprised a prolonged CL (that lasted 85 days with a diestrus length of 75 days); nor the anestrus periods were considered. For each examination, it was found a mean of 5.33 ± 1.57 follicles at the left ovary and 5.49 ± 1.25 at the right ovary (p>0,05) (Table 1).

Table 1. Mean cycle, estrus, diestrus and anestrus lengths for all groups and the total population and number of follicles found per ovary from September 1st until April 30th. § - correspondent to diestrus periods during the transitional periods; * - Mean diestrus length excluding the diestrus correspondent to the prolonged CL. Mean ± SD.

	Mean cycle length (days)	Mean estrus length (days)	Mean diestrus length (days)	Mean non-ovulatory period length (days)	Mean number of measured follicles per ovary
Anestrus (Group A)	24.13±3.03§	6.95±2.59§	16.94±4.42§	130.00±30.22	Left: 5.10±1.53 Right: 5.88±1.53
Silent estruses (Group SE)	25.00±3.03	5.67±1.75	19.33±3.87	N/A	Left: 5.65±2.10 Right: 5.73±2.37
Persistent CL (Group CL)	36.7±22.9 27.4± 3.80*	7.50±1.00	29.2±22.7 20.0±3.70*	N/A	Left: 2.68±0.45 Right: 3.72±0.34
Estrus, with acceptance	24.41±4.50	7.23±3.52	17.42±4.48	N/A	Left: 5.19±1.69 Right:

behavior (Group E)					5.58±1.63
Total Population	25.54±8.04 24.69±3.95*	7.18±3.68	18.89±8.21 18.03±4.30*	130.00±30.22	Left: 5.33±1.57 Right: 5.49±1.25

§ Data from cycles that were observed during transitional periods. *Excluded values from the cycle of jenny 15 that contained a prolonged CL. N/A – not applicable

Jennies' preovulatory follicles had a mean diameter of 38.02 ± 5.37 mm with the largest follicle registered measuring 56.82 mm and the smallest 26.21 mm. Between the last two examinations of each cycle, dominant follicles diameter variation was 1.57 ± 4.20 mm. The mean maximum diameter of the pre-ovulatory follicles during the non-breeding season was 34.14 ± 9.58 mm. Mean number of days between divergence and ovulation was 7.97 ± 3.31. The largest follicle at the moment of deviation had 20.15 ± 4.92 mm in diameter. When a second follicle became dominant, its mean diameter at the moment of deviation was 19.04 mm (Table 2). There were 3 ovulation failures, all in the anestrus group, two during spring transitional period and one during a vernal transitional period (Group A).

Table 2. Mean divergence day and mean follicular diameter, mean interval between divergence and ovulation, number of follicles found per ovary. Mean ± SD.

	Mean diameter of largest follicles (F1 and F2) at divergence	Days from divergence until ovulation	Mean preovulatory follicle diameter (mm)	Mean variation of preovulatory follicular diameters between two last examinations (mm)
Anestrus	F1: 23.97 ± 7.79 F2: 18.28 ± 2.68	9.50 ± 4.55	39.71 ± 4.46	0.50 ± 2.61
Silent estruses	F1: 19.04 ± 3.99 F2: 19.56 ± 3.16	6.86 ± 1.82	37.6 ± 5.68	2.17 ± 4.47
Persistent CL	F1: 19.86 ± 3.26 F2: 17.78 ± 3.55	9.00 ± 2.24	41.6 ± 3.74	3.45 ± 6.55
Estrus, with acceptance behavior	F1: 20.02 ± 4.19 F2: 19.81 ± 3.65	8.00 ± 3.65	37.19 ± 5.08	0.90 ± 3.37
Total Population	F1: 20.15 ± 4.92 F2: 19.04 ± 3.21	7.97 ± 3.31	38.02 ± 5.37	1.57 ± 4.20

In a total of 89 registered ovulations, 56.2% were from the left ovary and the remaining 43.8% from the right ovary. There were 47 single ovulations (69.1%), 20 double ovulations (29.4%) and one registered triple ovulation (1.47%), out of 68 ovulatory cycles. Amid all the multiple ovulations (n=21), synchronous ovulations come about in 52.4% of cases in opposition to asynchronous ovulations which occurred in the remaining 47.6% (Table 3).

Table 3. Left vs right ovary ovulations, single double and triple ovulations; asynchronous vs synchronous ovulations.

	Left vs Right ovary ovulations	Single, double and triple ovulations	Asynchronous vs Synchronous Ovulations
Anestrus	Left: 63.6% Right:36.4% (n=22)	Single: 77.8% Double: 22.2% (n=18)	Synchronous: 75% Asynchronous: 25% (n=4)
Silent estruses	Left: 78.3% Right:21.7% (n=23)	Single: 72.2% Double: 27.8% (n=18)	Synchronous: 60% Asynchronous:40% (n=5)
Persistent CL	Left: 30% Right:70% (n=10)	Single: 20% Double: 60% Triple: 20% (n=5)	Synchronous: 60% Asynchronous: 40% (n=5)
Estrus, with acceptance behavior	Left: 48.5% Right:51.5% (n=33)	Single: 73.1% Double: 26.9% (n=26)	Synchronous: 28.6% Asynchronous: 71.4% (n=7)
Total Population	Left: 56.2% Right:43.8% (n=89)	Single: 69.12% Double: 29.41% Triple: 1.47% (n=68)	Synchronous: 52.38% Asynchronous: 47.62% (n=21)

Overall, 1.03 ± 0.18 primary waves were observed per cycle. Of the 60 cycles studied, 18.3% and 25% presented major and minor secondary waves, respectively. During the non-breeding season, each cycle presented a mean of 1.04 ± 0.19 waves. Of the cycles that presented minor waves (n=29), 65.52% correspond to anestrus periods. Of the cycles that presented major secondary waves (n=13), 46.15% correspond to anestrus periods. The mean interval of days between peaks of successive waves for the entire study population was 20.06 ± 12.16 days (Table 4). For all the considered groups the mean interval between wave peaks is shorter than the mean cycle length. Several minor waves were observed mainly during non-breeding season in the anestrus group. Considering days between October 31st and March 30th, jennies of Group A had a mean

of 3.40 ± 2.61 minor waves from the ones of the remaining groups. For the whole population, 1.75 ± 2.3 minor waves were observed during the same period of time. The total number of observed minor waves was significantly higher (p= 0.027) in jennies of Group A during the period of time mentioned above.

Table 4. Number and type of waves present through September 1st until April 30th. Interval between waves. Mean ± SD. P – Major primary wave, M – Major secondary wave, m – minor wave.

	Number of primary and secondary waves (major and minor) per cycle	Mean interval between follicular wave peaks (days)
Anestrus	P:1.00 ± 0.00 M:0.55 ± 0.82 m:1.73 ± 2.33	19.00 ± 11.87
Silent estruses	P:1.00 ± 0.00 M:0.06 ± 0.24 m:0.17 ± 0.38	24.79 ± 16.54
Persistent CL	P:1.2 ± 0.45 M:0.2 ± 0.45 m:0	20.83 ± 11.20
Estrus, with acceptance behavior	P:1.04 ± 0.20 M:0.19 ± 0.40 m:0.27 ± 0.53	17.00 ± 7.94
Total Population	P:1.03 ± 0.18 M:0.22 ± 0.49 m:0.48 ± 1.20	20.06 ± 12.16

For Group A, minor waves had its largest follicle reaching a maximum of 21.44 ± 5.73 mm in diameter. Mean day of maximum diameter is shown in Table 5 for each minor wave. Groups SE, CL and E had minor waves starting to regress after mean day 9.80 ± 3.68 of each cycle when its largest follicle was 23.65 ± 5.14 mm in diameter. During the period of anestrus of jennies of Group A, 3 major secondary waves were observed, with its days of divergence expressed in Table 5. Groups SE, CL and E had divergence occurring at day 8.50 ± 10.89 of the cycle. Major secondary waves had divergence occurring at mean diameter 26.57 ± 7.31 and 25.76 ± 5.19 for group A and groups SE, CL and E together, respectively.

Table 5. Maximum diameter and respective day in minor waves, mean day of divergence and largest follicle's diameter of major secondary waves. Mean ± SD. M1, M2, M3 - successive major secondary waves observed during the same period; m1, m2, m3, m4, m4 m5, m6- successive minor waves observed during the same period.

	Mean maximum follicle diameter (minor waves) (mm)	Day of the cycle of maximum follicle diameter (minor waves)	Mean follicle diameter at divergence (major secondary waves) (mm)	Mean divergence day (major secondary waves)
Group A	21.44 ± 5.73 (n=17)	m1: 11.25 ± 7.89 (n=4) m2: 39.00 ± 27.13 (n=4) m3: 52.50 ± 37.44 (n=4) m4: 56.00 ± 17.58 (n=3) m5: 88.00 ± 4.24 (n=2) m6: 104.5 ± 9.19 (n=2)	26.57 ± 7.31 (n=5)	M1: 66.60 ± 54.49 (n=5) M2: 100.50 ± 40.31 (n=2) M3: 135 (n=1)
Groups SE, CL and E	23.65 ± 5.14 (n=4)	9.80 ± 3.68 (n=4)	25.76 ± 5.19 (n=4)	8.50 ± 10.89 (n=4)

Jennies from groups SE, CL and E had, from October 31st until March 30th, had a higher number of observed follicular waves when comparing to jennies from group A (6.29 ± 2.43 vs. 4.4 ± 2.07). This difference was not significant (p=0.191).

2. Follicular profiles in jennies that underwent anestrus periods

In this group of 5 jennies, there was a continuous follicular development with successive follicles growing and regressing without ever achieving ovulation (Figure 8). Cycles corresponding to vernal and spring transitions presented a mean estrus and diestrus length of 8.33 ± 5.04 and 16.94 ± 4.42 days, respectively. Anestrus lasted an average of 130.0 ± 30.2 days. The longest anestrus period lasted 157 days and the shorter one 83 days. Of all transitional periods, the longest estrous periods lasted 20 and

23 days and the shortest estrus lasted 2 days. Diestrus ranged from 6 to 26 days. There were 5.1 ± 1.79 follicles at the left ovary and 5.88 ± 1.53 follicles at the right ovary in each examination.

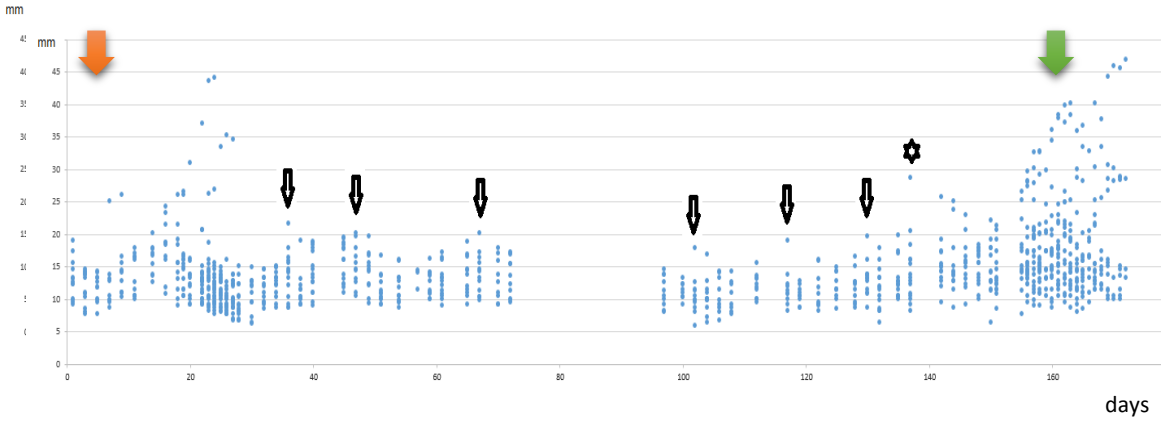

Figure 8. Follicles registered of Jenny 3 (Group A) from 5th October to 25th March. After the last ovulation of the reproductive season, at 31st October, with a double asynchronous ovulation, 6 secondary minor waves can be observed (arrows) till the beginning of March, followed by a major ovulatory wave, that culminated with a double asynchronous ovulation by day 28 and 31 March. Between the last minor wave and the first primary wave (flower) it seems to have ensued a major secondary wave. The gap in the figure corresponds to a period when data on follicle size was not collected. Orange arrow: vernal equinox. Green arrow: spring equinox.

There were 14 single ovulations (77.8%) and 4 multiple ovulations (22.2%). Of the 4 multiple ovulations, 75% of them were synchronous and the remaining 25% were asynchronous. Considering total number of ovulations, 63.6% were from the left ovary and 36.4% from the right ovary. The preovulatory follicle of these jennies' cycles measured 39.71 ± 4.46 mm in diameter, ranging from 47.51 mm to 31.36 mm. Specifically for the first ovulations after an anestrus period (n=3), the preovulatory diameter was 42.6 ± 4.13 mm. Three of the five jennies failed to ovulate and two of these three failed ovulations during spring transitions. The other failed ovulation corresponds to a cycle that occurred before the anestrus period, during vernal transition. The preovulatory follicles had a mean diameter positive variation of 0.50 ± 2.61 mm between the last two examinations.

In ovulating cycles, one primary wave per cycle was observed. Of all cycles analyzed in group A, 36.36% presented major secondary waves and 54.55 % presented minor waves. Among the 5 jennies displaying anestrus, all presented minor waves, and 60%

presented major secondary waves. Minor waves were significantly more frequent (p=0.027) for group A when compared to the other groups between October 31st and March 30th. Groups E, SE and CL had a higher, but not significantly higher, number of follicular waves per cycle (p=0.191) during the same period of time.

The mean interval of days between peaks of successive waves for all jennies was 19.00 ± 11.87 days. The mean maximum diameter of the follicular waves during the non-breeding season was 29.26 ± 10.69 mm. Primary waves (n=9) had divergence occurring 9.50 ± 4.55 days before ovulation. Mean diameter of the largest follicle at the moment of divergence was 23.97 ± 7.79mm. When a second follicle became dominant, its mean diameter was 18.28 at divergence.

During anestrus and transitional periods, 87.92% of all follicles have a diameter lower than 18mm. Of the remaining 12.08%, 51.68% of the follicles larger than 18 mm were present during estrus periods. Largest follicles in minor waves during anestrus reached 21.44 ± 5.73 mm in diameter.

3. Follicular patterns of jennies that kept their normal cyclicity

Jennies number 6, 7, 8, 9 and 13 kept their normal cyclicity, ovulating with estrus behavior year-round. These animals displayed a mean cycle length of 24.41 ± 4.50 days (ranging from 43 to 18 days), a mean estrus length of 7.23 ± 3.52 days (ranging from 21 to 3 days) and a mean diestrus length of 17.42 ± 4.48 days (ranging from 36 to 8 days). Jennies 6 and 13 presented particularly long diestrus lengths during the breeding and non-breeding season. A mean of 5.19 ± 1.69 and 5.58 ± 1.63 follicles were detected and measured per ultrasound exam in the left and right ovaries, respectively.

Of 33 preovulatory follicles, 48.5% were from the left ovary and the remaining 51.5% from the right ovary. All cycles considered (n=26), there were 73.1% single ovulations and 26.9% multiple (double) ovulations. Among the multiple ovulations (n=7), 71.4% were asynchronous and the remaining 28.6% were synchronous. Preovulatory follicles had a mean diameter of 37.19 ± 5.08 mm, with the largest preovulatory follicle measuring 56.82 mm in diameter and the smallest measuring 27.63 mm in diameter. Mean variation in diameter between the last two examinations was 0.90 ± 3.37 mm. In

jenny 7, during spring transition, two dominant ovulatory follicles emerged from two different primary waves in the same cycle, ovulating on the same day (Figure 9).

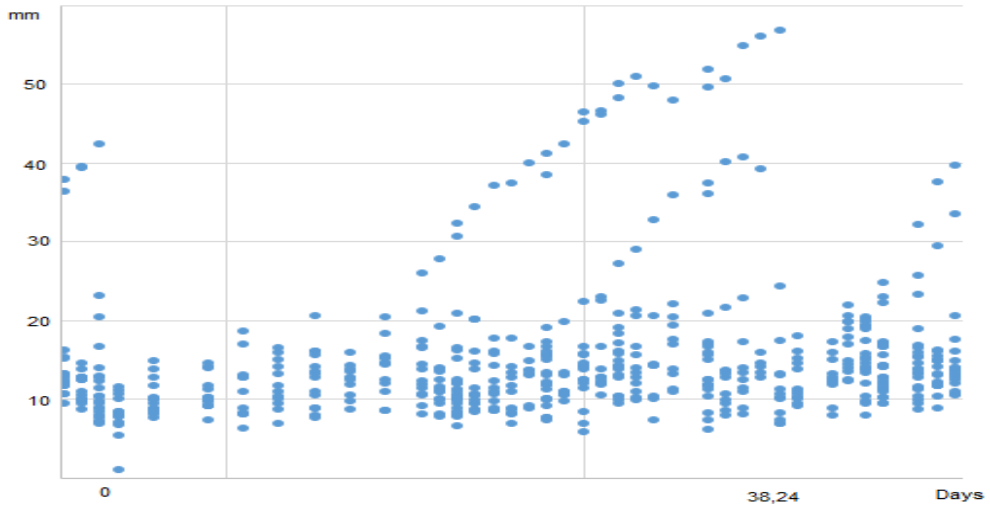

Figure 9. Estrous cycle of jenny 7 (Group E) with two dominant follicles originating from two different major primary follicular waves. Cycle lasting from 19th November to 27th December, with estrous period duration of 18 days.

Major secondary waves were detected in 19.23% (n=5) of the cycles, while 23.08% (n=6) of the cycles presented minor waves (Figure 10). The mean interval between peaks of successive waves, all these jennies considered, was 17.00 ± 7.94 days. The mean maximum diameter of the follicular waves during the non-breeding season was 35.64 ± 6.66 mm. Primary waves (n=26) had divergence arising 8.00 ± 3.65 days before ovulation. Mean diameter of the largest follicle of the wave at divergence was 20.02 ± 4.19 mm. When a second follicle of the primary wave became dominant, its mean diameter at divergence was 19.81 ± 3.65 mm.

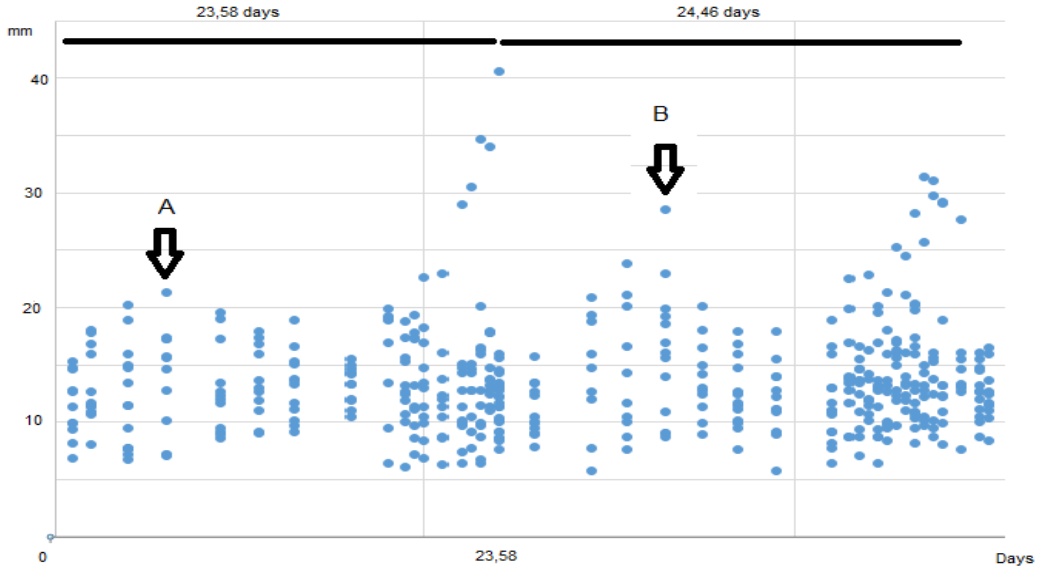

Figure10. Two consecutive estrous cycles of jenny 8 (Group E), from 3rd October to 22th November. In the first cycle, that lasted 23.58 days, from previous ovulation to ovulation of a single follicle from a major primary wave, after an estrous period of 6 days, it was detected a minor secondary wave (arrow A), with the largest follicle reaching 21.36 mm by day 6 of the cycle, before regressing. In the second cycle, that lasted 24.46 days, it was detected a major secondary wave with a follicle reaching 28.57 mm, by day 9 of the cycle. This second cycle ended with an asynchronous ovulation of two follicles from the left ovary, measuring 28.18 and 29.86 mm, on their largest diameter.

4. Follicular profiles of the jennies with silent estruses

Jennies that displayed silent estruses (jenny 2, 10 and 14) presented a mean cycle length of 25 ± 3.03 days (20 to 33 days). Mean estrus and diestrus length were 5.67 ± 1.75 and 19.33 ± 3.87, respectively. Estrus length ranged from a maximum of 8 days to a minimum of 1 day. Diestrus ranged from a maximum length of 27 days to a minimum of 13 days. A mean of 5.65 ± 2.1 and 5.73 ± 2.37 follicles were found and measured in each examination at the left and right ovaries, respectively. In a total of 23 preovulatory follicles, 78.3% ovulated from the left ovary and 21.7% ovulated from the right ovary. There were 13 single ovulations (72.2%) and 5 double ovulations (27.8%). Amid multiple ovulations (n=5), 40% were considered asynchronous and 60% were synchronous.

Mean diameter of preovulatory follicles was 37.6 ± 5.68 mm, ranging from 52.27 to 26.21 mm. Mean variation in diameter of the preovulatory follicles between the last two examinations was 2.17 ± 4.47 mm. Considering only the cycles that presented with silent estruses (n=11), mean preovulatory diameter was 34.26 ± 4.06 mm (ranging from 40.43 to 26.21 mm).

All cycles (n=18) presented one major primary wave. Only one major secondary wave was observed in one jenny, which corresponds to only 5.56% of the cycles (Figure 11). Minor waves occurred in 16.67% (n=3) of the cycles, two in jenny 2 and one in jenny 10. The mean interval of days between peaks of successive waves, for these jennies was 24.79 ± 16.54 days. The mean maximum diameter of the follicular waves during the non-breeding season was 37.33 ± 9.29 mm. In major primary waves, divergence occurred at day, 6.86 ± 1.62 days before ovulation. Mean diameter of the largest follicle at divergence was 19.04 ± 3.99. When a second follicle became dominant, its dimeter at divergence was 19.56 ± 3.16 mm.

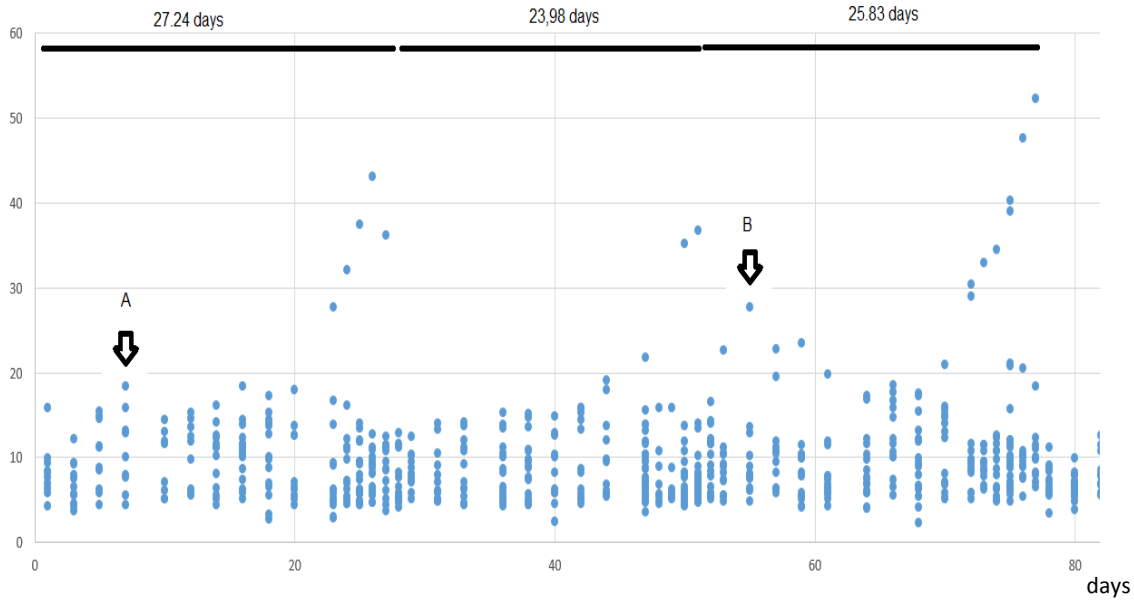

Figure 11. Three consecutive estrous cycles of jenny 2 (Group SE), from 9th October to 25th December. the first cycle, that lasted 27.24 days, ended with the ovulation of a single follicle from a major primary wave, after an estrous period of 5 days, it was detected a minor secondary wave (arrow A), with the largest follicle reaching 18.36 mm by day 7 of the cycle, before regressing. In the second cycle, that lasted 23.98 days, no secondary waves were detected. This second cycle ended with the ovulation of a follicle from the left ovary, measuring 36.76 mm, on the last measurement before ovulation. The third

shown cycle had again a minor secondary wave (Arrow B), peaking 4 days after previous ovulation, ending with the ovulation of a very large ovulatory follicle (52.27 mm).

5. Follicular profiles of the jenny that presented a persistent CL

Of all the studied cycles (n=5), the female donkey that had a persistent CL (n=1) had a mean cycle length of 36.7 ± 4.50 days (21 to 83 days), mean estrus length of 7.5 ± 1.0 days (9 to 6 days) and mean diestrus length of 29.2 ± 22.7 days (75 to 14 days). Excluding the cycle that encompassed a prolonged CL, the remaining cycles had a length of 27.4 ± 3.80 days (31 to 21 days) and diestrus lasted 20.0 ± 3.7 days (23 to 14 days). The longest registered cycle lasted 83 days and corresponds to the one with a persistent CL. This cycle presented an estrus and diestrus length of 8 and 75 days, respectively. A mean of 2.68 ± 0.45 and 3.72 ± 0.34 follicles were recorded and measured during each ultrasound exam in the left and right ovaries, respectively.

Of the 5 cycles observed, one had a simple ovulation and the remaining had multiple ovulations. Of the 4 multiple ovulations, there were 3 double ovulations and one triple ovulation. Of all ovulations, 3 were asynchronous, and the remaining were considered to be synchronous. There were 25% of ovulations occurring from the left ovary and the remaining 75% ovulated from the right ovary, of a total of 12 preovulatory follicles. Mean diameter of preovulatory follicles was 41.6 ± 3.74 mm, ranging from 50.23 mm to 37.32 mm. In between the last two examinations the preovulatory follicular diameter had a mean variation of 3.45 ± 6.55 mm.

Jenny number 15 had 6 primary waves (1.2 ± 0.45 waves per cycle) and one major secondary wave. The mean interval of days between peaks of successive waves was 20.83 ± 11.20 days. The mean maximum diameter of the follicular waves during the non-breeding season was 41.32 ± 7.41 mm. Primary waves had divergence occurring 9.00 ± 2.24 days before ovulation. Mean diameter of the largest follicle of the primary wave at divergence was 19.86 ± 3.26 mm. When a second follicle became dominant, its mean diameter was 17.78 ± 3.55 mm.

V. Discussion

1. General cycle characterization and seasonal effects

Differently from what is known in horses (Snyder et al., 1979; Aurich, 2011), nevertheless in agreement with what was observed by Ginther et al. (1987), the majority of the jennies in the present study did not display a marked follicular inactivity during winter months. A more profound knowledge on this subject might allow a better use of artificial insemination (AI) and other assisted reproduction techniques during winter months, an increase and better distribution of parturitions throughout the year, which can help increasing endangered species' population and a better care of fewer newborns at each season. Also, a homogeneous yearly distribution of pregnant jennies can be of economic importance for milk industry.

Of the fifteen jennies studied, jenny 1 was excluded due to insufficient data collection. In the studied population of *Miranda* donkey breed jennies, 33.3% of the studied females kept their normal reproductive activity, a lower rate than the 50% previously reported by Ginther et al. (1987) but higher than the 15.4% reported by Henry et al. (1987). Three out of the fourteen jennies presented a variable number of silent estruses (Group SE) during the non-breeding season; one had a persistent CL (Group CL) and five out of the fourteen went through a period of seasonal anestrus (Group A). However, jennies in seasonal anestrus kept having continuous follicular activity, with emergence of minor waves, though with the absence of large dimension follicles and ovulations.

Ginther et al. (1987) and Henry et al. (1987), reported longer interovulatory intervals and longer estrus during winter months, when compared with the breeding season, as well as the occurrence of anestrus periods for a variable number of jennies. No effects on diestrus length were reported by these authors. Different conclusions were presented for the *Martina Franca* donkey breed, reporting decreased estruses and increased diestrus during winter months, when compared to summer months (Contri et al., 2014). *Miranda* jennies, during the breeding season, present a 23.8 ± 0.55 days interovulatory interval, with estrus and diestrus lasting 6.65 ± 0.30 and 17.9 ± 0.46, respectively (Quaresma and Payan-Carreira, 2015).

When comparing results of this previous study with the ones now obtained for the non-breeding season, it is concluded that slightly longer interovulatory intervals were verified

among all Groups (A, SE, CL and E) agreeing with what was reported by Ginther et al. (1987) and Henry et al. (1987). The same results were obtained with estrus length, with the exception of the Group that presented silent estruses (Group SE), with a shorter mean estrus length of 5.67 ± 1.75 days. Diestrus was longer than what is reported for the breeding season, with the exception of jennies of Group A, that presented shorter diestrus periods during transitional periods and jennies from Group E, in wich diestrus was very similar to breeding season mean diestrus length, agreeing with results on *Martina Franca* donkey breed (Contri et al., 2014).

The recurrent long diestrus lengths observed in jennies 6 and 13 during the breeding and non-breeding season suggest it is rather an individual characteristic than a season effect. Jenny 15 presented a mean diestrus length of 29.2 ± 22.7, with a high variability among its cycles due to the occurrence of a persistent CL that led to a prolonged diestrus (75 days) and interovulatory interval. The occurrence of this disturbance in the estrous cycle of jennies has already been described (Ginther et al., 1987).

The mean anestrus length of Group A (130.00 ± 30.22 days) is longer than what was reported by Ginther et al., (1987) - 39 to 72 days. Differences might be due to different methodologies, differences between the population selected for the studies, individual variations among the selected individuals, among other aspects, like nutrition and BCS variation (Donadeu and Watson, 2007). The results obtained in the present study also differ from the ones reported for the same studied population by Quaresma et al. (2015), due to the fact that in the calculation of the mean anestrus length interval, the diestrus periods after the last ovulations during vernal transition phases, with an active CL, were not encompassed.

All ovulations considered (n=89), 56.2% were from the left ovary and the remaining 43.8% were from the right ovary. This higher occurrence of ovulations from the left ovary was also described during the breeding season in *Miranda* breed donkeys (Quaresma and Payan-Carreira, 2015), Catalonian jennies (Taberner et al., 2008) and Tropical jennies (Lemma et al., 2006c), though never significantly. These results suggest either that ovulations from the left or population size is not big enough to reach significant results.

Single ovulations were more frequent, with 69.12% single ovulations in contrast to 29.41% and 1.47% of double and triple ovulations, respectively. Single ovulations were more frequent in all jennies with the exception of jenny 15, with only 1 single ovulation, 3 double ovulations

and 1 triple ovulation. Jenny 15 was also the only one presenting a triple ovulation during the period of time studied. The results herein obtained report a higher occurrence rate of multiple ovulations than the 14.3% of multiple ovulations published by Vandeplassche et al., (1981). However, this work was published before ultrasonography was used on reproductive exams, and these different methods can lead to different results. More recent studies for the breeding season in females of the *Miranda* donkey breed present higher rates of multiple ovulations than the ones found in our study: 36.36% and 6.06% for double and triple ovulations, respectively (Quaresma and Payan-Carreira, 2015). A study with Catalonian jennies mentioned rates of 55.66%, 42.45% and 1.89% of single, double and triple ovulations, respectively (Taberner et al., 2008). Another study in Spain found an occurrence rate of multiple ovulations of 49.2% (Galisteo and Perez-Marin, 2010), while, for Mammoth jennies, multiple ovulations occurred in 61.11% of the estrous cycles (Blanchard et al., 1999).

When comparing the prevalence of multiple ovulations incidence during the breeding and non-breeding season, higher values are found for the breeding season, agreeing with Galisteo and Perez-Marin (2010), which have mentioned an increase of multiple ovulations at the beginning of the breeding season. A lower occurrence of multiple ovulations during the non-breeding season might be an advantage, as there is a lower probability of twin pregnancies. Repeatability of multiple ovulations among jennies, initially suggested by Vandeplassche et al. (1981) and already confirmed in the same *Miranda* breed jennies' population for the breeding season (Quaresma. and Payan-Carreira, 2015) seems to also be present during the non-breeding season.

During our study, 52.38% of the multiple ovulations occurred synchronously (with less than 24 hours of interval between them) and the remaining 47.62 % happened asynchronously (in different days). Catalonian jennies presented 41.18% synchronous ovulations (Taberner et al., 2008) and *Miranda* breed jennies had 50% of multiple ovulations occurring within the same 24h during breeding season (Quaresma and Payan-Carreira, 2015). In mares, Ginther and Pierson (1989) found no relevant differences between the number of synchronous and asynchronous ovulations. Differences between groups were observed with jennies that kept normal reproductive activity and the jenny with a persistent CL presenting an opposite tendency from the population, with higher rates of asynchronous ovulations. Overall, our results are similar to the one's obtained for the same breed during the breeding season, which

might indicate that time between ovulations of the same cycle might not be influenced by season.

Anovulatory follicles have been described by some authors as occurring with no regard to season (Cuervo-Arango et al., 2009); while others reported an expectable higher frequency of these structures during transitional and deep anestrus periods (Nequin et al., 2000; Donadeu and Ginther, 2002; Watson et al., 2002). Also, Paccamonti (2012) reported them to occur in 11.9% of the cycles during the breeding season of mares and 22.2% of the cycles during transitional periods. Kebede et al. (2012) reported 17.9% of the observed estrus periods ending up in anovulatory follicles in jennies in Ethiopia. In our study, during the considered period, 5% of the ovulations ended with an anovulatory follicle. The three ovulation failures were all observed in Group A; two corresponded to a major wave at the end of an anestrus period and the third took place during vernal transition. Our results agree with the ones reported by other authors (Nequin et al., 2000; Watson et al., 2002, Donadeu and Ginther, 2002) but with a lower rate of anovulatory follicles.

The occurrence of anovulatory follicles at the end of spring transition in some jennies suggests that it could be that in females that undergo an anestrus period the first emerging large follicle should be disregarded for mating. Also for the first cycles of the breeding season, ultrasound Doppler exams might be useful to evaluate vascularity and follicular viability near ovulation day. Lefranc and Allen (2003), reported a mean maximum size of 48.1 ± 8.0 mm of anovulatory follicles in mares. The observed maximum diameter in our study was 39.39 ± 5.98 mm, a lower diameter than the one presented above but similar to the one Kebede et al., (2012), presented for a group of jennies in Ethiopia. A possible role of BCS in relation to first ovulations of the season was found to exist in mares (Gastal et al., 2004).

In each ultrasound session, a mean of 5.33 ± 1.57 and 5.49 ± 1.25 follicles were measured in the left and right ovary respectively. During the present study, in all groups, the right ovary had always a higher number of follicles measured which can be probably explained by an influence of the hand used by the operator during the ultrasound exams. Jenny number 15, which presented a persistent CL, has the lowest number of measured follicles, 2.68 ± 0.45 and 3.72±0.34 follicles in the left and right ovaries, respectively. A possible explanation is the

continued presence of a large CL, as equids CL characteristics can turn follicle identification harder and influence its results (Kimura et al., 2005).

Lemma et al. (2006c) observed a lower number of follicles present during the periods of low sexual activity of tropical jennies: 7.3 follicles per ovary in the dry season, in opposition to 9.6 and 11.3 follicles per ovary in the short and long rainy seasons, respectively. As for Egyptian jennies, a mean of 6.40 ± 0.26 follicles per ovary were found with a bigger number of small follicles present rather than medium and large size follicles (Abdoon et al., 2014). Similarly, a recent study in Ethiopia found a mean of 5.45 ± 2.3 follicles per ovary, with no significant differences between left and right ovaries for a group of jennies in Ethiopia; it also verified a bigger occurrence of smaller follicles rather than larger follicles during the season of low sexual activity (Kebede et al., 2012). Our results are very similar to the ones found for jennies in Ethiopian and Egyptian jennies. A comparison with the breeding season of the *Miranda* donkey breed is not possible due to the absence of published data on this subject.

In Group A, during anestrus and transitional periods, 87.92% of all follicles had a diameter lower than 18mm. Of the remaining 12.08%, 51.68% of the follicles larger than 18 mm were present during the estrus of transition periods. Vandeplassche et al. (1981) suggested an initial low number of large follicles, an increasing number of medium follicles around 7 days before ovulation that would start to decrease together with the number of small follicles until ovulation occurs. Further data analysis would be necessary to determine whether this occurs in the *Miranda* donkey breed.

For the breeding season, it is known that the ovulatory follicle of *Miranda* jennies measures around 38.4 mm (Quaresma and Payan-Carreira, 2015), not very different from what was obtained in our study: 38.02 ± 5.37. It was noticed an apparent tendency of the groups that showed seasonal effects (Groups A, ES and CL) for having larger preovulatory follicles than Group E. A tendency for large mean ovulatory follicles diameters at the onset of the breeding season was confirmed, as it was already reported for *Miranda* breed jennies (Quaresma et al., 2015). In relation to other donkey breeds, all the available data is related with the breeding season. Our results present a smaller mean ovulatory diameter than the mean 46.3 mm ovulatory follicle of Catalonian jennies (Taberner et al., 2008), 41 mm of a group of jennies in Egypt (Derar and Hussein, 2011) and 44 mm in a group of Ethiopian jennies (Kebede et al.,

2012). However, our results show evidence of a larger mean ovulatory diameter than the ones of tropical jennies during the rainy season (Lemma et al., 2006c) and Anatolian jennies (Kalender et al., 2012), with 37.8 mm and 32.25 mm, respectively.

As described for mares (Ginther et al., 2008) and donkeys (Taberner et al., 2008; Quaresma and Payan-Carreira, 2015) in the breeding season, preovulatory follicles stop growing and might even regress in diameter near ovulation day. This was verified among a considerable number of ovulations during winter months. However, follicles that had a considerable growth between these two examinations influenced the mean results obtained with the mean variation between the last two examinations, with a recorded final growth of 1.57 ± 4.20 mm. A consistent slowdown in follicular growth rate during the last days before ovulation was not determined in the course of our study.

Divergence occurred in Groups A, SE, CL and E, at days 9.50 ± 4.55, 6.86 ± 1.82, 9.00 ± 2.24 and 8.00 ± 3.65 days before ovulation, respectively. Overall, divergence occurred 7.97 ± 3.31 days before ovulation, when the largest and second largest follicles were 20.15 ± 4.92 and 19.04 ±3.21 mm in diameter. Deviation occurs in the mare when the largest and second largest follicles are 21 to 23 mm and 19 mm in diameter, respectively (Ginther, 2000; Beg and Ginther, 2006). Quaresma and Payan-Carreira (2015), during breeding season, mention that the largest follicle measures 19.18 mm in diameter and deviation occurrs around 9 days before ovulation in *Miranda* breed jennies; Kebede et al. (2012) reported the largest follicles near deviation measuring 17.9 mm in diameter for jennies in Ethiopia, also during the breeding season. Our results for groups A, CL and E for the non-breeding season are similar to what is reported for this breed of donkeys; jennies from Group SE presented the shortest intervals of days from divergence to ovulation. Deviation occurred when follicles had similar dimensions to the ones reported for *Miranda* jennies, larger than the ones observed for Ethiopian jennies and smaller than what is observed in mares, with the exception of Group A that presented larger follicles at deviation.

2.Follicular waves

Ultrasonography exams were taken approximately every other day from the beginning of October to the end of April of the following year. This methodology does not allow following each follicles identity, being that possible only for the largest follicle or follicles that, after deviation became dominant, as Kebede et al. (2012) has already done, based on Ginther (1986).

Not following each follicle's identity leads to the unfeasibility of determining when exactly does a follicular wave emerges, because of the impossibility to assert if a follicle is part of an emerging wave or of a regressing wave of the previous cycle (Pierson and Ginther, 1987; Ginther, 1993) being this period of time classified as an "area of ambiguity" (Ginther and Bergfelt, 1992). This "area of ambiguity" was observed during our study when either a minor or major wave started to regress or other waves started to emerge (Figure 12 and 13).

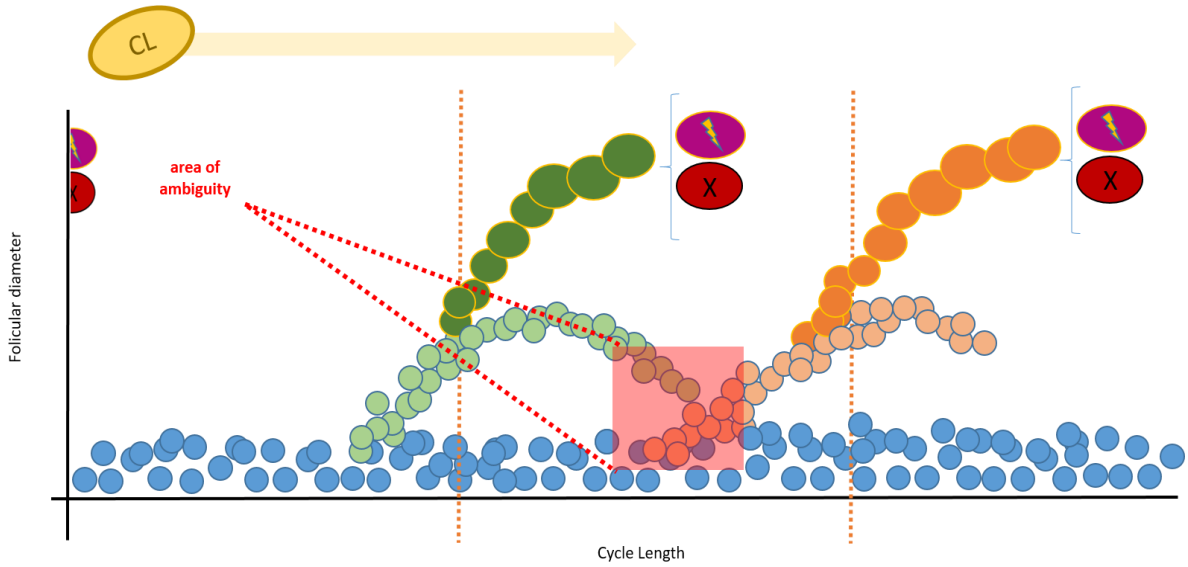

Figure 12. Representation of a cycle with a major secondary (**green dots**) and primary wave (**orange dots**), respectively and the resultant area of ambiguity (**red square**). A wave pattern observed during transitional periods or during the non-breeding season in jennies that do not cease its normal cyclicity. In **yellow** is represented luteal activity subsequent from a previous ovulation. **Orange vertical dotted lines** represent wave deviation moment. In **blue** is represented the follicular pool from where follicles are recruited for wave emergence. Dominant follicles might either ovulate (**pink dot**) or regress (**red dot**).

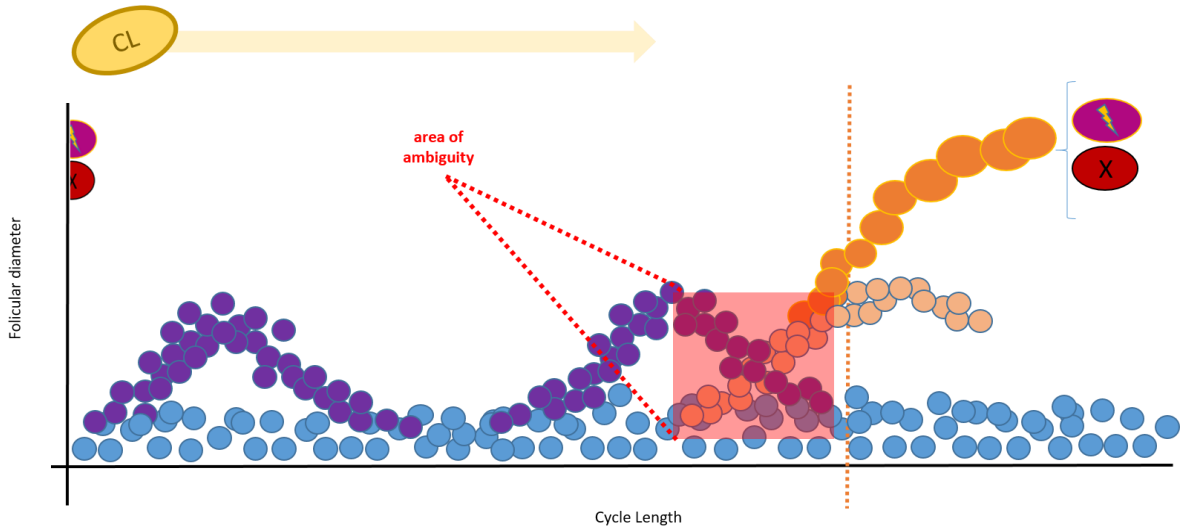

Figure 13. Representation of a cycle with minor waves (**purple dots**) and a primary wave (**orange dots**), respectively and the resultant area of ambiguity (**red square**). A wave pattern observed during transitional periods or during the non-breeding season in jennies that do not cease its normal ciclicity. In **yellow** is represented luteal activity subsequent from a previous ovulation. The **orange vertical dotted line** represents wave deviation moment. In **blue** is represented the follicular pool from where follicles are recruited for wave emergence. The dominant follicle might either ovulate (**pink dot**) or regress (**red dot**).

For this reason, the number of follicular waves, in particular minor waves, that generally present follicles of smaller dimensions, might be hard to calculate. Despite its limitations, this type of study has already been previously done with success in mares, using statistical tests to evaluate follicular waves (Ginther and Bergfelt, 1992; Donadeu and Pedersen, 2008). It was verified by Bergfelt and Ginther (1992) that FSH surges precede the emergence of any type of follicular wave. The regular quantification of this hormone might, in this way, be a solution to identify wave emergence when a non-identity method is used as it was used in cattle (Adams et al., 1992; Bo et al., 1995; Bodensteiner et al., 1996; Ginther et al., 1996) and sheep (Ginther et al., 1995; Duggavathi, 2005) to study ovarian follicular waves. However, it would increase the cost of the study, which in some cases limits the possibility of its use.

During the whole analyzed period of time, even in anestrus jennies, follicles kept developing in what appears to be cohorts of follicles with a variable level of organization, in agreement with Irvine (1981), that found that, for mares, when more than one follicular wave had developed, the last one was also the one that originates the ovulatory follicle or follicles. Nowadays it is accepted for mares a one-wave model, in which, per cycle generally only one follicular wave develops and originates the ovulatory follicle (Figure 14) during the breeding season (Ginther, 1992; Sirois et al., 1989). After transitional periods, 1 to 2 follicular waves are expectable (Ginther and Bergfelt, 1992). Our results point an average of 1.04 ± 0.20 waves per cycle. Derar and Hussein (2011) reported the existence of only one wave per cycle during the breeding season of jennies in Upper Egypt but the existence of 2 to 3 follicular waves per cycle has also been reported in jennies (Kebede et al., 2012). Our results also agree with Sánchez-Berná and Pérez-Marín (2000), that have reported the existence of major and minor follicular waves during the Andalusian jennies' estrous cycle.

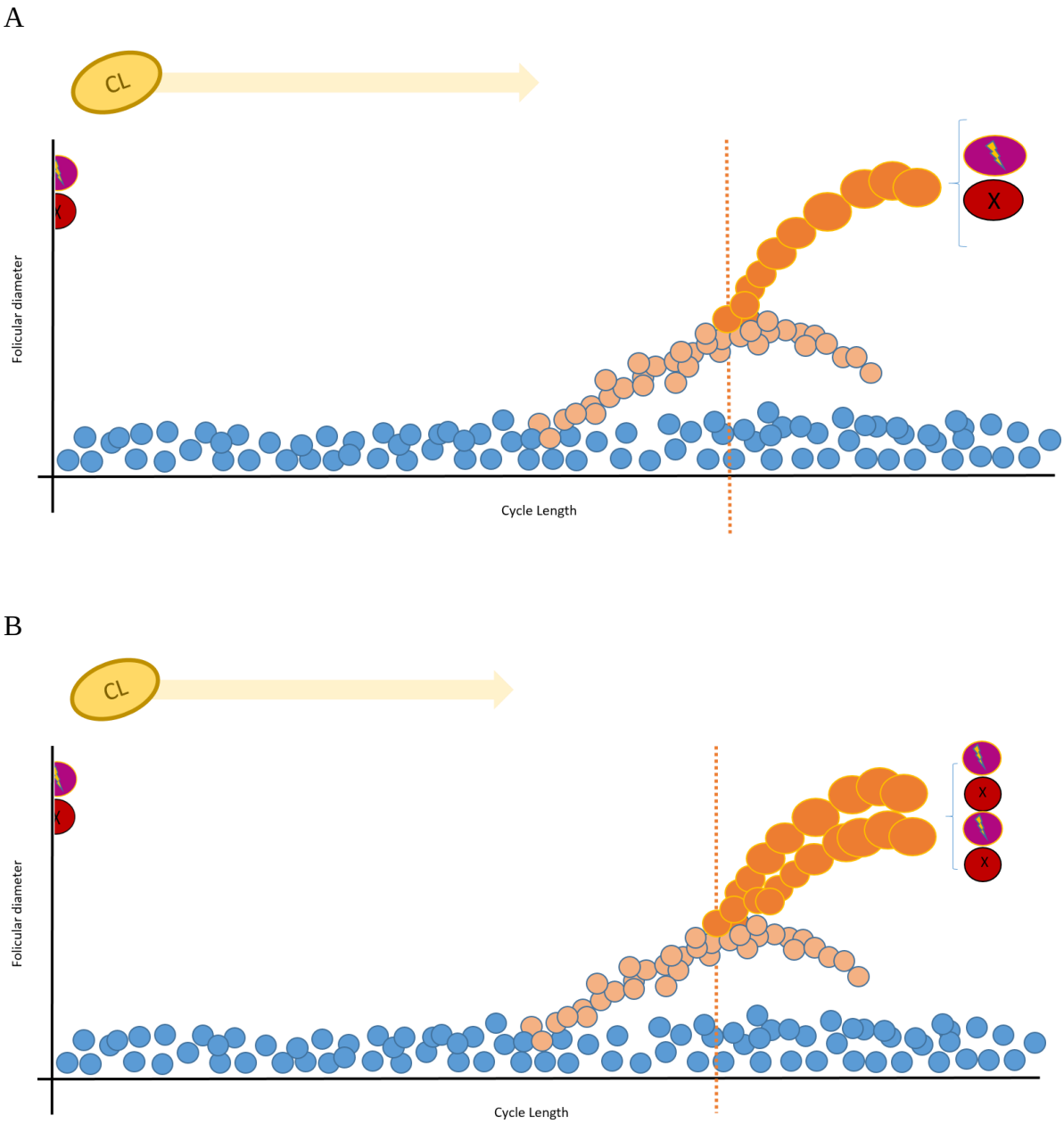

Figure 14. Most frequent one-wave pattern found in mares and jennies. Single ovulation (A) and double ovulation (B). In **yellow** a CL and the correspondent luteal activity is represented following the previous cycle's ovulation. In **blue** is represented the small diameter follicular pool from where follicular recruitment happens. In orange is represented a primary follicular wave. The **vertical orange dotted** line marks deviation, after what subordinate follicles regress (**light orange**) and the one that reached dominance (**orange**) keeps developing until it either ovulates (**pink dot**) or fails to ovulate and regresses (**red dot**).

Overall, of the observed cycles (n=60), 18.3% presented major secondary waves and 25% of the cycles presented minor waves. An average of one major primary wave per cycle would be expectable. However, our result of 1.03 ± 0.18 major primary waves per cycle can be explained by a jenny that presented a double ovulation from two consecutive primary waves that emerged both during the second half of the cycle. Jennies from Groups SE, CL and E had a higher, but not significant (p=0.191), number of follicular waves, in the same period of time, comparing to Group A, as it was expected. A larger sample would probably lead to statistical significance. Of all cycles that presented minor waves (n=29), 62.52% correspond to anestrus periods of five jennies (Group A). Considering the cycles that presented major secondary waves, 46.15% occurred during anestrus periods of jennies from Group A. However, marked differences among groups were observed. The results obtained suggest a tendency for a higher occurrence of minor waves during anestrus periods (Figure 15). Considering the days between October 31st and March 30th minor waves were significantly more frequent in jennies of Group A when comparing to the ones of the other groups (p=0.027).

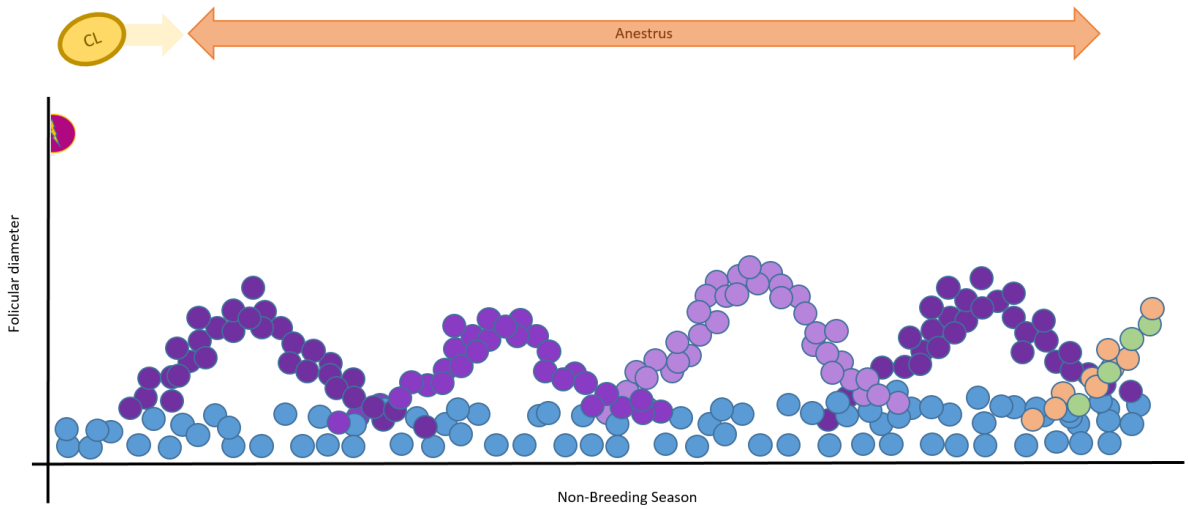

Figure 15. Representation of the most frequent follicular dynamics pattern within jennies that underwent an anestrus period (Group A). The vernal transition period's ovulation (**pink dot**) gives origin to the last active CL (yellow dot and arrow) before the onset of the anestrus period (**orange arrow**). During anestrus follicular recruitment occurs from the small follicle pool (**blue dots**). A variable number of minor waves (**purple dots**) develop with follicles never reaching divergence nor preovulatory dimensions. At the end of the anestrus period a major wave (secondary or primary wave) starts to develop (**orange** and **green** dots) marking the beginning of spring transition.

Our results show that minor waves were more common on the anestrus jennies during the non-breeding season than in the other groups. Also, major secondary waves seemed to be more common near the onset of the breeding season agreeing with what Ginther (1993) reported for the mare's estrous cycle. As it was previously mentioned, our method of data collection might have influenced our results, with the number of minor waves being underestimated (Ginther, 1993). These results indicate that, not only a slowdown in cell proliferation occurs, but also interference in the deviation process ensues, as there is continued wave emergence, but the larger follicles fail to acquire dominance. The occurrence of major secondary waves during anestrus indicates that in some jennies, deviation can occur even though ovulation fails.

The interval between consecutive wave peaks was determined as an attempt of estimating the mean interval, in days, between the emergences of two consecutive waves and overcoming our method's limitation of not being able to determine the day of wave emergence. Overall, a follicular wave had its largest follicles reaching maximum dimensions every 20.06 ± 12.16 days. Group E had the shortest mean interval, 17.00 ± 7.94 days. This points out to the fact that the jennies normally cycling also were the ones with the smallest intervals between waves, probably a sign of a more active hypothalamus-hypophysis-gonadal hormonal system. Also, the mean interval between wave peaks is shorter than the observed cycle lengths as during many cycles more than one wave occurred.

In mares, only minor waves can be found developing in the ovaries during anestrus season; follicles of minor waves are usually not bigger than 21 mm during deep anestrus. On the other hand, major secondary waves are usually more common during fall and spring transitions (Figure 16). Also during spring transition, follicular diameter increase in relation to what is observed during deep anestrus (Ginther, 1990; Donadeu and Pedersen, 2008). Our results revealed minor waves in Group A with a maximum follicular diameter similar to what is described for mares (21.44 ± 5.73 mm), though jennies of Groups SE and E presented a mean maximum diameter higher than the one reported for mares (23.56 ± 5.14 mm). Group CL presented no minor waves during the considered period of time. The mean day of the cycle when the largest follicle of minor waves reached its maximum dimensions was also evaluated; calculating the mean difference of days between successive wave peaks it is possible to conclude that minor waves reached a peak on average 18.65 ± 11.42 days apart.

Considering that follicles grow at a similar pace between waves, probably it can be said that the pattern of minor wave emergence during non-breeding season in anestrus jennies is repeated on average, every 18 to 19 days.

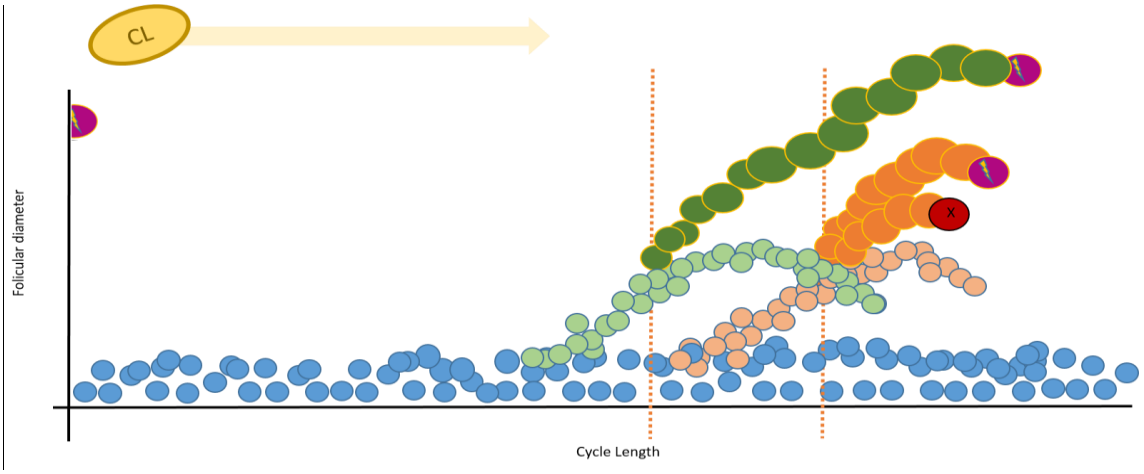

Figure 16. Representation of a double ovulation observed in jenny 7. The cycle begins with an active CL **(yellow dot and arrow)** originated from the previous cycle's ovulation. Luteal activity decreases until emergence of the first primary wave, that emerges during the second half of the cycle **(light green dots).** The second primary wave **(light orange dots)** emerges. The orange dotted vertical lines signal divergence of both secondary and primary waves. Dominant follicles keep developing **(green** and **orange).** Ovulations are marked by **pink dots**. With a **red dot** is signed the second dominant follicle of the primary wave that did not reach ovulation and regressed. **Blue dots** indicate the small diameter follicle pool from where follicles are recruited before any wave emergence.

Also evaluated were the mean day of divergence of major secondary waves and the diameter of the largest follicles at divergence. Overall the maximum follicular diameter of minor waves was smaller than the diameter at divergence of the largest follicles of the major secondary waves, evidencing that follicles of minor waves did not reach a size adequate for divergence to occur. Mean maximum diameter of major secondary waves was smaller in all groups than the maximum diameter of the ovulatory follicles of primary waves, agreeing with what Ginther and Bergfelt (1992) reported.

Jenny number 7 had a double ovulation, with each ovulatory follicle developing from a different primary wave (Figure 16). Divergence of both waves occurred with 16 days of

interval and ovulations occurred with 3 days of interval. The first primary wave had one follicle becoming dominant and ovulating from the right ovary and the second primary wave had one ovulatory follicle from the left ovary and a second dominant follicle in right ovary that regressed, never reaching ovulation. This phenomenon has been described in mares (Ginther, 1993; Ginther and Bergfelt, 1992), though for donkeys, until today, the authors found no mention of it in previous reports. Only one triple ovulation was observed during our entire study (Figure 17). It corresponded to the last ovulation before the onset of the breeding season of jenny 15.

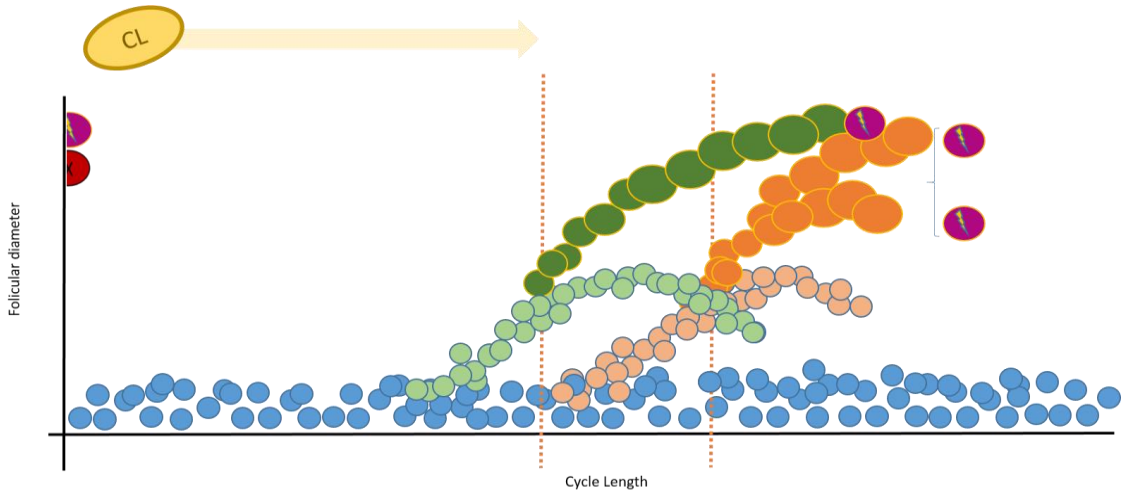

Figure 17. Representation of a triple ovulation observed in jenny 15. The cycle begins with an active CL **(yellow dot and arrow)** originated from the previous cycle's ovulation. A major secondary wave emerges during the first half of the cycle **(light green dots).** Luteal activity decreases until emergence of the primary wave **(light orange dots)** during the second half of the cycle. The orange dotted vertical lines signal divergence of both secondary and primary waves. Dominant follicles keep developing **(green** and **orange)**. Ovulations are marked by **pink dots**. **Blue dots** indicate the small diameter follicle pool from where follicles are recruited before any wave emergence.

VI. Conclusion

This study aimed to characterize follicular wave dynamics during the non-breeding season of the *Miranda* breed jennies. In general, the results were in agreement with what is published for the reproductive season, for donkeys in general and, more specifically, with data published on this particular breed.

A major finding was that jennies in seasonal anestrus kept having continuous follicular activity, with emergence of minor waves, though with the absence of large dimension follicles and ovulations. Ovulation failures of large dimension pre-ovulatory follicles were also only found among jennies that underwent a period of seasonal anestrus. Less follicles were found per ovary than what was reported for the breeding season agreeing, suggesting a breakdown in ovarian activity during winter months. Single ovulations were more common and agreed with previous results on *Miranda* breed jennies, that reported a tendency for it to be associated with each jenny individually. Ovulations from the left and right ovaries had similar number of occurrences as well as synchronous and asynchronous ovulations.

All jennies presented continued follicular development. Minor waves were more frequent during anestrus periods. In general, maximum diameter of the largest follicles of minor waves were smaller than the diameter at divergence of the largest follicles of major waves. Multiple ovulations occurred either from the same primary wave, from a major secondary wave and a primary wave or from two distinct primary waves. Our method of data collection did not allow following each follicle's identity nor determination of wave emergence. Minor waves were probably underestimated.

By following the identity of the largest follicles it was possible to characterize follicular dynamics in *Miranda* breed jennies during the non-reproductive season. Future application of an identity method and the comparison between results would be important to validate this method, as it was done in mares. Also studying the influence of BCS and age on the occurrence of each type of follicular waves would be interesting as well as the induction of ovulation by administration of drugs.

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