

# Reproductive responses of anestrus ewes to the introduction of rams

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## Abstract

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This thesis summarises and discusses results of studies concerning the ovarian and endocrine responses of anestrus ewes to the ram effect, the relation between the use of progestogen primings and the reproductive response of anestrus and cyclic ewes to the ram effect, and the endocrine and testicular changes in rams used to stimulate anestrus ewes.

Ovarian responses to the ram effect (monitored ultrasonographically) were highly variable. Ewes responded with two luteal phases (short and normal, respectively), delayed ovulations (Days 57) followed by normal or short luteal phases, luteinization of non-ovulatory follicles, luteinized follicular cyst and no luteal phase. There was no relation between the growth status of the largest follicle present when rams were introduced and the ovarian responding pattern of anoestrus ewes to the ram effect.

Similar results in estrus incidence and fertility were obtained after using intravaginal sponges containing medroxyprogesterone acetate (MAP) for 6, 9, or 13 days. Priming with sponges containing 20, 40, or 60 mg of MAP for 6 days gave similar reproductive results. Six-day primings with sponges impregnated with MAP or fluorogestone acetate, or intravaginal devices containing progesterone (CIDR) were equally effective in improving the response to the ram effect. A single administration of 2.5 mg of MAP 1, 3, or 5 days before the introduction of the rams concentrated estrus in ewes 17 to 20 days later. In MAP-primed ewes, the endocrine pattern of the induced follicular phase of ewes that came into estrus was similar to a normal follicular phase, in ewes that ovulated without expressing estrus no significant increase in estradiol-17 $\beta$  or decrease in FSH was observed.

The ram effect does not seem to affect the ovarian response of cyclic ewes during the mid-breeding season.

Rams used to stimulate anestrus ewes show an increase in LH and testosterone concentrations beginning at 12 h after joining, and high concentrations are maintained while estrus ewes are present and mating takes place in the flock. The response of rams to estrus ewes may be at least part of the mechanism by which anestrus ewes submitted to the ram effect express maximum reproductive response when ewes in estrus are introduced together with the rams.

Key words: LH, FSH, estradiol-17 $\beta$ , progesterone, testosterone, follicular dynamics, ultrasonography, seasonal anestrus, *Ovis aries*

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Todos los imperios del futuro van a ser imperios del conocimiento, y solamente serán exitosos los pueblos que entiendan cómo generar conocimientos y cómo protegerlos; cómo buscar a los jóvenes que tengan la capacidad para hacerlo y asegurarse que se queden en el país. Los otros países se quedarán con litorales hermosos, con iglesias, minas, con una historia fantástica; pero probablemente no se queden ni con las mismas banderas, ni con las mismas fronteras, ni mucho menos con un éxito económico.

Albert Einstein

***To Damiana***

***To Pamela***

***To Mariana***



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# Appendix

## Papers I-VI

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Ungerfeld, R., Pinczak, A., Forsberg, M. & Rubianes, E. 2002. Ovarian and endocrine responses of Corriedale ewes to "ram effect" in the non-breeding season. *Canadian Journal of Animal Science* 82, 599-602.

II. Ungerfeld, R., Dago, A.L., Rubianes, E. & Forsberg, M., 2003. Response of anestrus ewes to the ram effect after follicular wave synchronization with a single dose of estradiol-17 $\beta$ . *Submitted*.

III. Ungerfeld, R., Suárez, G., Carbajal, B., Silva, L., Laca, M., Forsberg, M. & Rubianes, E. 2003. Medroxyprogesterone priming and response to the ram effect in Corriedale ewes during the nonbreeding season. *Theriogenology* 60, 35-45.

IV. Ungerfeld, R., Pinczak, A., Forsberg, M. & Rubianes, E. 1999. Response of Corriedale ewes to the "ram effect" after priming with medroxyprogesterone, fluorogestone, or progesterone in the non-breeding season. *Acta Veterinaria Scandinavica* 40, 299-305.

V. Ungerfeld, R., Carbajal, B., Rubianes, E. & Forsberg, M. 2003. Endocrine and ovarian changes in response to the ram effect in medroxyprogesterone primed Corriedale ewes during the breeding and the non-breeding season. *Submitted*.

VI. Ungerfeld, R., Silva, L., 2003. Ewe effect: endocrine and testicular changes in adult and young Corriedale rams used for the ram effect. *Animal Reproduction Science*, in press.

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## General introduction

La fortuna de un relato no está solo en la habilidad del que lo escribe, sino quizás igualmente en la experiencia heredada de quien lo lee.

Robert Louis Stevenson

## Sheep production in Uruguay

Uruguay, with an area of native and improved pastures of 15.2 million ha (82% of the country's area), is one of the world's leading sheep-producing countries (Cardellino, Salgado & Azzarini, 1994). The mean annual rainfall is 1100 mm, distributed over 85 days/year, but with important variability within years and seasons; mean temperatures range from 12° in July to 24° in January (Cardellino, Salgado & Azzarini, 1994). Most farms produce both sheep and cattle, but with important differences in the sheep:cattle ratio according to the region of the country. The northern and central parts of the country (3.4 million ha) have the highest ratio, with sheep as approximately 30% of the overall livestock, due to the characteristics of the soil (basaltic soil).

During the late 1990s, sheep constituted the second largest livestock sector throughout the world (Morand-Fehr & Boyazoglu, 1999). The Uruguayan flock was approximately 25 million in 1991 but decreased to 12 million in 2001 (Censo General Agropecuario, 2000), a trend observed also in the rest of the world as a consequence of declining international wool prices (Figure 1). Wool production decreased 18% in Uruguay and 35% throughout the world as a whole during the last decade (Cardellino, 2003).

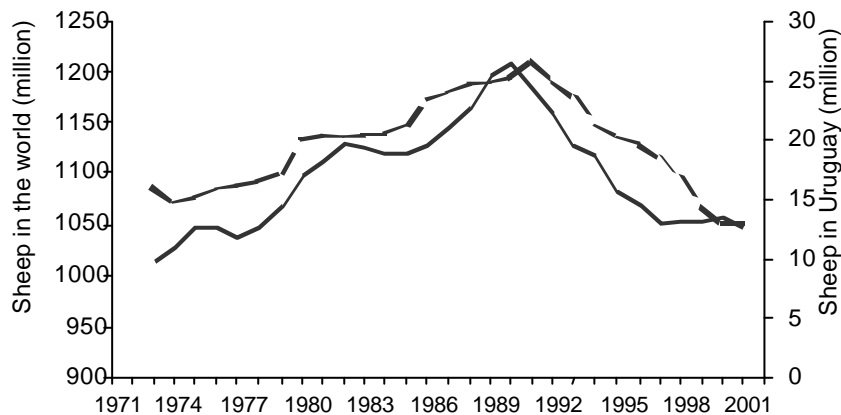


Figure 1. Overall number of sheep in the world (—) and in Uruguay (----) between 1971 through 2001 (data from FAO, 2003).

In Uruguay, approximately 65% of the farmers breed Corriedale sheep (data from 1998; R. Cardellino, personal communication). Corriedale is a dual-purpose breed and was originally developed in New Zealand and Australia during the late 1800s by crossing Lincoln or Leicester Longwool rams with Merino females (Majjala, 1997). Most of the world's stock

of Corriedale is found in South America, but the breed, being the second most significant in the world, is also raised throughout Asia, North America, and South Africa (Breeds of Livestock, 2002). The Corriedale flock in Uruguay is one of the biggest in the world (Cardellino, 1995). Other breeds in Uruguay are Australian Merino (14%), Polwarth (9%), Merilin (5%), Romney Marsh (1%), and crossbreeds (4%) (Data from 1998; R. Cardellino, personal communication).

### **Production strategies and reproductive management of sheep**

Recently some new production alternatives have begun to develop, which may be useful to compensate for the negative effects of declining wool prices (Azzarini, Oficialdegui, & Cardellino, 1996). Traditionally, meat has been a by-product of wool production (Cardellino, 1988), but in recent years lamb production has started to develop as an alternative or complement to wool production (Azzarini, 1992, 1995). It has also been common for sheep to be bred in a very extensive way, with long breeding periods from March to May (autumn) (Azzarini, 1992), and lambing between August and October.

Reproductive seasonality represents a natural adaptation that provides important advantages for birth and offspring survival and development, as lambing coincides with good weather and maximum availability of forage. But seasonal breeding is also an important barrier to flexible management of sheep (Haresign, 1992), when taking into account market and economic requirements (Lindsay & Thimonier, 1988). In meat-producing systems it is important to develop techniques to induce ewes to conceive at precise times of the year, frequently outside the breeding season (Lindsay & Thimonier, 1988). Out-of-season lambing provides several advantages such as premium prices or accelerated reproductive systems. In Uruguay, lamb prices change according to the season, with the highest prices being found during winter and spring (Figure 2). Uruguay has good regional opportunities for meat export but a continuous supply should be offered (Vázquez Platero & Picerno, 1997). Thus, it is interesting for farmers to obtain births during late summer and early autumn in order to supply the market with lamb meat throughout the year.

The Corriedale breed has a marked seasonal reproductive pattern (Rodríguez Iglesias et al., 1993). In Uruguay, the breeding season ranges from February to June (SUL, 1994; Perdigón, Sosa & Cavestany, 1997). This period is similar in that in other areas at the same latitude (Buenos Aires, Argentina: Sánchez, Alberio & Burges, 1994) or slightly higher latitudes (Bahía Blanca, Argentina: Irazoqui & Menvielle 1982; Hamilton, New Zealand: Cummins, Spiker & Wilson, 1992). Consequently, in order to produce winter lambs, techniques for out-of-season estrous induction have to be developed.

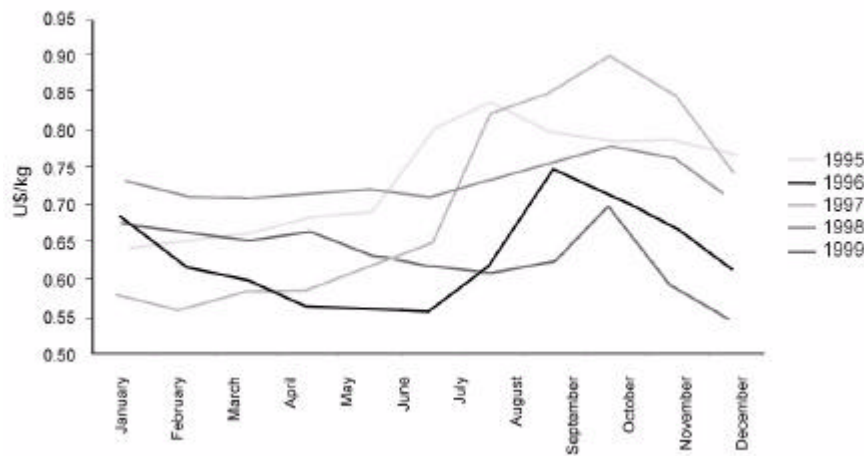


Figure 2. Meat prices of heavy lambs in Uruguay by month of the year (modified from Parma, 2001).

## Background

There are several techniques to induce estrus in anestrus ewes (for review, see Smith et al., 1989). Briefly, there are pharmacological techniques (use of progestogen + gonadotrophin, melatonin) or management techniques (selection according to onset of breeding season or introduction of rams to stimulate reproductive cyclicity – the ram effect).

The use of progestogen + eCG, which mimics the hormonal pattern of a normal estrous cycle, may be effective in obtaining a high percentage of ewes in estrus (Ungerfeld & Rubianes, 1999a, 2002). However, this technique is far from being cost-effective under local market conditions. As well, the use of progestogens is prohibited in some jurisdictions such as the USA and the European Union, as a consequence of increasing pressure from consumers for hormone-free animal production systems (Martin, 2001). The selection of animals according to their individual seasonal pattern is an effective alternative (Notter, 2001; Vincent, McQuown & Notter, 2000), but it takes a long time to obtain early-lambing flocks.

The ram effect may be a useful and suitable tool, considering its cost, which is negligible. The response to the ram effect may also be used as a good indicator as criteria for selecting individual animals to develop an early-lambing flock (McQueen & Reid, 1988). Results obtained to trigger the reproductive systems of anestrus ewes with the ram effect are at least similar to those obtained with hormonal treatments (Boly et al., 2000; Crosby & Murray, 1988; Martemucci et al., 1984).

### *A theoretical analysis of the biological significance of seasonality and the ram effect*

To understand the mechanisms underlying physiology, we should attempt to relate them to what we know about how those mechanisms may have developed during the evolution of the species. We do not know if mammals originally had an annual cyclic pattern of reproduction that evolved towards different seasonal patterns, or if different patterns have always existed among ancestral mammals (Rowlands & Weir, 1984). In any case, seasonal patterns of reproduction should be a consequence of selective processes related to best conditions for parturition and nursing. To measure the appropriate time for conception, animals would then be forced to use different environmental cues such as photoperiod (sheep, horse), temperature (ground squirrel), humidity (chinchilla), rainfall, and improved nutrition (rodents) (Lindsay, 1988). Social cues may also trigger the onset of the breeding season in wild and feral ruminants.

Domestic animals display some differences in their reproductive physiology compared with their wild ancestors. Primitive cattle have short breeding seasons (Reinhardt, Reinhardt & Reinhardt, 1986), probably initiated in response to a decreasing photoperiod. However, during the domestication process, cattle have been selected to breed throughout the year (Rowlands & Weir, 1984) and show very little annual reproductive variation. A similar pattern can be observed in swine: wild sows have a seasonal reproductive pattern (Ahmad et al., 1995; Mauget, 1981), but the domestic pig breeds throughout the year (Asdell, 1964). As well, wild horses have a shorter breeding season compared with domesticated horses (Rowlands & Weir, 1984). The reproductive pattern of the sheep is a bit different: wild sheep have a short breeding season, and most developed breeds retain a seasonal reproductive pattern (Setchell, 1992).

The high degree of reproductive synchrony observed in wild and feral female sheep may be at least partially a consequence of male introduction and other social interactions (Signoret, Cognié & Martin, 1984). The social structure throughout the year is similar in wild and feral sheep breeds (Soay: Grubb & Jewell, 1973; Rocky Mountain Bighorn [*Ovis canadensis canadensis*]: Geist, 1971; Punjab Urial [*Ovis orientalis punjabiensis*]: Schaller & Mirza, 1974; Mouflon: McClelland, 1991), and in farmed breeds (Romney: Knight, Ridland & Litherland, 1998). Outside the rutting period, social groups are comprised of several females with their offspring, or of males exclusively joining in small groups (Stricklin & Mench, 1987). When male offspring become mature they disperse from the female group (Shackleton & Schank, 1984). It has been suggested that sexual segregation is caused by differences in movement patterns and ruminating/foraging schedules caused in turn by different nutritional demands due to sexual dimorphism in body size (Ruckstuhl, 1998). However, wethers (castrated male sheep) remain together or with females (Jewell, 1997), suggesting that the testis – probably through androgens – is involved in the segregation. As the time of breeding approaches, males join the female groups. Nudging, blocking, and rubbing (Jewell, 1976) and aggressive behavior (Lincoln & Davidson, 1977) by males begin before females come into estrus, probably as a consequence of the earlier activation of the male reproductive system (increased LH pulsatility, increasing FSH and testosterone concentrations; for review, see Lincoln & Short, 1980). In sheep, natural joining may trigger – through the ram effect – an earlier onset of the breeding season, as has been seen in ewes allowed to be in permanent contact with rams (Eldon, 1993; O'Callaghan et al., 1994). In

addition, there is evidence that blinded ewes in permanent contact with rams (Legan & Karsch, 1983) or pinealectomized ewes in a flock with rams and intact ewes (Wayne, Malpoux & Karsch, 1989) have a more synchronous onset of the breeding season than those that remain isolated.

The rut period causes an increase in energy expenditure of rams (Jewell, 1997), so what may be the significance of beginning the reproductive season before ewes are cyclic? Moreover, what sense does it make to have a mechanism where males trigger the female reproductive system? The mechanism may be especially important in breeds that display a very short breeding season (e.g., Soay: 1 to 3 estrous cycles, Grubb & Jewell, 1973). Moreover, late conception in Rocky Mountain sheep during the breeding season increases lamb and ewe mortality (Hogg, Hass & Jenni, 1992). The stimulus may also promote an advancement of puberty in females, which may allow them to increase their reproductive success throughout their lifetime (Bérubé, Festa-Bianchet & Jorgenson, 1999). The period from joining of males and females until the peak of estrus is also useful for males to sort out hierarchical ranks (Jewell, 1976). Estrous synchronization allows different males to mate different females, decreasing the risks of inbreeding and of the reduction of genotype variation that would result.

Overall, the available information suggests that there is an evolutionary mechanism underlying the reproductive response of domestic ewes to the introduction of rams.

#### *What do we know about the ram effect, and how have we learned it?*

The social stimulus has been studied in several species. Table 1 summarizes the reproductive effects that males may induce in females. Although not completely understood, a male stimulus on female reproductive physiology may also exist in caribou (Adams et al., 2001), musk deer (Green, 1987), camels (Claus et al., 1999), wild boars (Delcroix, Mauget & Signoret, 1990), and dogs (Naaktgeboren & Van Straalen, 1983). In elephants, pheromones (chemical substances that are produced by animals and stimulate particular behavioral responses in other individuals from the same specie) seem to play a very important role in reproduction (Rasmussen & Schulte, 1998). In humans, some authors claim a direct effect of the male on ovulation (Veith et al., 1983), and some of the described effects are possibly caused by pheromones (reduction in variability in cycle lengths: Cutler et al., 1986; changes in LH pulsatility: Preti et al., 2003). However, the existence of pheromones (Hays, 2003) and the function of the vomeronasal organ in humans (Witt et al., 2002) are controversial issues.

Table 1. Species in which stimulating effects have been demonstrated and the main effects produced by males on female reproductive physiology. Such effects have been widely demonstrated in rodents, of which selected examples are presented here.

<b>Species</b>	<b>Effect</b>	<b>Reference</b>
Sheep	Estrous induction in seasonal anestrus	Underwood, Shier & Davenport., 1944
	Shortening of postpartum in ewes	Wright, Geytenbeek & Clarke, 1984
Goat	Advancement of puberty in lambs	O’Riordan & Hanrahan, 1989
	Estrous induction in seasonal anestrus	Chemineau, 1987
	Advancement of puberty in goats	Mellado, Olivas & Ruiz, 2000
	Synchronization of puberty onset	Amoah & Bryant, 1984
Cattle	Advancement of postpartum rebreeding	Custer et al., 1990
	Advancement of postpartum rebreeding in relation to body condition	Alberio et al., 1987
	Advancement of puberty	Rekwot et al., 2000
Red deer	Advancement of the breeding season	Moore & Cowie, 1986
	Advancement of puberty	Fisher, Meikle & Johnstone, 1995
Eld’s deer	Advancement of estrus and the LH peak	Hosack et al., 1999
Reindeer	Advancement of onset of breeding season	Shipka, Rowell & Ford, 2002
Moose	Synchronization of the breeding season	Whittle et al., 2000
	Induction of ovulation	Miquelle, 1991
Antelope	Modification of estrous cycle duration	Skinner, Cilliers & Skinner, 2002
Oryx	Advancement of puberty	Blanvillain et al., 1997
Impala	Advancement of the breeding season	Skinner, Jackson & Marais, 1992
Blesbok	Increase in length of breeding season	Skinner, Jackson & Marais, 1992
Pig	Advancement of first postpartum ovulation	Walton, 1985
	Advancement of puberty	Brooks & Cole, 1970
Mare	Stimulation of ovulation	Bour & Palmer, 1985
Rabbit	Possible increase in litter size	Rodríguez De Lara et al., 2003
Opossum	Estrous synchronization	Perret & Ben M’Barek, 1991
	Estrous induction	Jackson & Harder, 1996
Meadow vole	Advancement of puberty	Baddloo & Clulow, 1981
Prairie vole	Advancement of puberty	Carter et al., 1980
Bank vole	Induction of ovulation	Clarke & Hellwing, 1977
Rat	Induction of ovulation	Johns et al., 1978
Wild guinea pig	Induction of ovulation	Weir, 1971

Since the publication by Underwood, Shier & Davenport (1944), several studies about the ram effect have been performed. Scientific interest in the subject steadily increased until the 1980s, when most reports were published (Figure 3). The number of papers per year parallels the world population of sheep (Figure 1), with a decrease in the beginning of the 1990s. This suggests that research on the ram effect has been closely related to production interests and industry funding (for review, see Martin, 1995). Most reports have come from Australia, New Zealand, and Europe (mainly France and the United Kingdom). Latin America, which has had between 8% and 11.2% (data from FAO, 2003) of the world’s sheep stock during last 30 years, has contributed 7.5% of the overall reports on the subject.



This world distribution of research is directly related to breeds used. Although many breeds have been studied (Table 2), more than 60% of the information was obtained from Merino, Romney, and their crossbreeds.

The response of a ewe to the ram effect depends on the strength of the stimulus and the receptiveness of the ewe. There are ewes that will not respond regardless of the strength of the stimulus (e.g., breeds with a strong seasonal pattern). On the other hand, some ewes will respond to a very light stimulus (e.g., breeds with a light/shallow anestrus close to the onset of the breeding season).

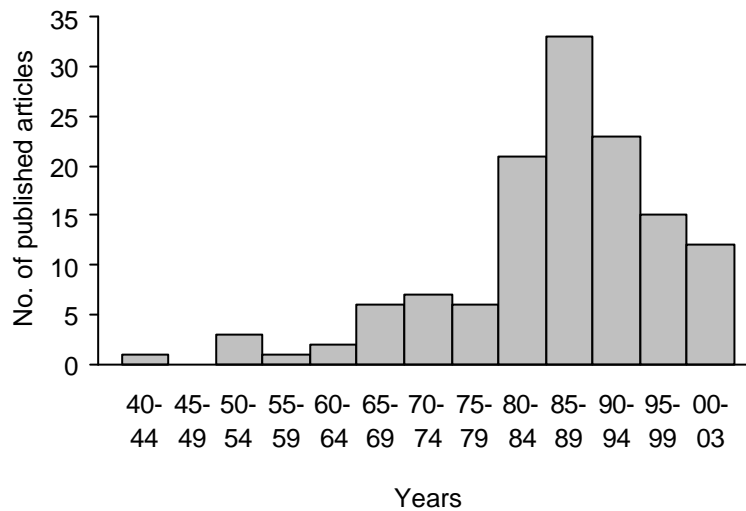


Figure 3. Number of articles about the ram effect published in international journals, including proceedings of the Australian and New Zealand Societies of Animal Production. (Reviews and the articles included in this thesis are not considered.)

### Factors associated with the stimulus

Many experiments have studied the female response to the ram effect, but few of them determined the importance of different characteristics of the ram. In goats, the reproductive condition of the buck seems to be the limiting factor determining the response of anestrus does to the male effect (Flores et al., 2000). The ram stimulates through pheromones, visual, and behavioral/tactile cues that act in a synergistic way. Several experiments have provided different, and sometimes contradictory, information about the importance of the different cues (Cohen- Tannoudji et al, 1989; Knight & Lynch, 1980a; Pearce & Oldham, 1988), but this may be a consequence of using ewes in different status of “receptiveness.”

Table 2. Sheep breeds in which the ram effect has been studied and country where the experiments have been performed.

<b>Breed</b>	<b>Location</b>	<b>Reference</b>
Altamura	Italy	Toteda et al., 1990
Appenninica	Italy	Lucidi, Barboni & Mattioli, 2001
Aragonesa	Spain	Abecia, Forcada & Zuñiga, 2002
Awassi	Syria	Kassem, Owen & Fadel, 1989
Barbarine	Tunisia	Lassoued et al., 1997
Belclare	Galway	Hanrahan & O'Riordan, 1990
Berrichon	France	Cognié et al., 1980
Boutsiko	Greece	Peclaris et al., 1999
Churra Galega Brangancana	Portugal	Correia et al., 1999
Cigaja	Serbia	Stancic et al., 1987
Clun Forest	Iceland	Dýrmundsson & Lees, 1972
Columbia	USA	Wheaton, Windels & Johnston, 1992
Coopworth	New Zealand	Scott & Johnstone, 1994
Corriedale	South Africa	Lyle & Hunter, 1967
Djalonké Mossi	Burkina Faso	Boly et al., 2000
Dorset	USA	Nugent III, Notter & Beal, 1988
Finn crossbreed	USA	Wheaton, Windels & Johnston, 1992
Florina	Greece	Triantaphyllidis et al., 1997
Gallega	Spain	Hernández et al., 1992
Greek Mountain	Greece	Peclaris, Mantzios & Nikolaou, 1992
Hampshire	USA	Nugent III, Notter & Beal, 1988
Icelandic	Iceland	Eldon, 1993
Ile de France	France	Cohen-Tannoudji et al., 1989
Manchega	Spain	Gómez Brunet et al., 1995
Menz	Ethiopia	Mukasa-Mugerwa et al., 1994
Merino breeds		
Merino	Australia	Fulkerson, Adams & Gherardi, 1981
German Merino	South Africa	Hunter & Lishman, 1967
German Mutton Merino	Germany	Kaulfuß et al., 1997
Konya Merino	Turkey	Aksoy et al., 1994
Merino Australiano	Uruguay	Azzarini, 1996
Merino Dárles	France	Cohen-Tannoudji & Signoret, 1987
Merino Dohne	South Africa	Nowers, Coetzer & Morgenthal, 1994
Rambouillet	USA	Hulet et al., 1986
Morkaraman	Turkey	Yildiz et al., 2003
Ossimi	Egypt	Barkawi, Barghout & Abdelaal, 1990
Pelibuey	Mexico	Martínez Rojero et al., 1998
Perendale	New Zealand	Taylor & Andrewes, 1987
Poll Dorset	Australia	Hall, Fogarty & Gilmour, 1986
Préalpes-du-Sud	France	Chemineau et al., 1993
Rahmani	Egypt	Hassan et al., 1988
Romanov	France	Martin et al., 1985
Romney Marsh	New Zealand	Knight & Lynch, 1980a
Sarda	Italy	Molle et al., 1997
Scottish Blackface	UK	Robinson et al., 1991
Southdown	Australia	Atkinson & Williamson, 1985
Suffolk	Japan	Fukui et al., 1988
Swedish Landrace Finewool	Sweden	Gates et al., 1998
Swedish Pelt	Sweden	Korjonen, 1997
Targhee	USA	Cushwa et al., 1992
Tsigai	Slovakia	Margetin, Malik & Misun, 1989
Tuj	Turkey	Yildiz et al., 2002
Vlakhiko	Greece	Laliotis et al., 1997

### *Role of pheromones*

According to some reports, full contact is not necessary for ewes to respond to rams (Watson & Radford, 1960). The scent of wool and wax from intact rams is enough to obtain a response in terms of ovulation in ewes (Knight & Lynch, 1980a). However, information about the importance of scent is contradictory. Morgan, Arnold & Lindsay (1972) observed that ewes with impaired smell did not respond to rams, but a normal LH response was observed in ewes reported as without vomeronasal activity (Cohen-Tannoudji et al., 1989) or olfactory activity (Cohen-Tannoudji, Locatelli & Signoret, 1986). Wool and wax are the main source of the pheromones that are part of the ram effect (Knight & Lynch, 1980a). Pheromones produced by the buck can also stimulate LH pulse frequency (Over et al., 1990) and ovulation in anestrus ewes (Knight, Tervit & Lynch, 1983), although bucks are less effective than rams (McMillan, 1987). The pheromones secreted by the boar seem to be ineffective in ewes (Knight, Tervit & Lynch, 1983). It has been demonstrated that pheromone production in rams is controlled by androgens (Croker et al., 1982; Fulkerson, Adams & Gherardi, 1981; Signoret, Fulkerson & Lindsay, 1982). The pheromones are present in aqueous and petroleum-spirit extracts of wool and wax (Knight & Lynch, 1980b). They are produced by the skin, especially around the eyes (Martin, 2001). Cohen-Tannoudji, Einhorn & Signoret (1994) partially identified the components present in wool through a bioassay that measured the LH response of ewes. They used extracts from fleece and from the ante-orbital gland of rams, and determined that to obtain a maximum reproductive response in ewes several compounds are needed. Mainly the acid fraction from the extract (without compound identification) plus a combination of 1,2-diols is responsible for the pheromone component of the ram effect. The use of pheromones alone in anestrus ewes has given controversial results: in one study pheromones did not induce any changes in LH or FSH secretion (Schneider & Rehbock, 2003). In other investigations, the use of pheromones resulted in ovulation (Kaulfuß et al., 1997; Kaulfuß, Schenk & Süß, 2002) or in an increase in pregnancy rates of inseminated ewes (Milovanov, 1991).

### *Other stimulating cues*

Pearce & Oldham (1988) stimulated ewes with masks containing ram's wool – and thus probably pheromones – but the maximum reproductive response was obtained only with full contact between rams and ewes, suggesting that in some ewes with low “receptiveness” behavior/tactile cues are also needed. Moreover, some authors have suggested that other sensory cues may completely replace the pheromone stimulus (Cohen-Tannoudji, Locatelli & Signoret, 1986; Cohen-Tannoudji et al., 1989). Perkins & Fitzgerald (1994) demonstrated the importance of the sexual behavior of the rams. A higher number of ewes ovulated when put together with rams expressing high libido compared with ewes put together with rams expressing low libido, although testosterone levels of the rams appeared to be similar.

### *Breed and percentage of rams in the flock*

There is little information about using rams from different breeds; most experiments have been focused on Dorset and Romney rams (Table 3). Lindsay, Wilkins & Oldham (1992) observed more ewes in estrus when they used 3% or 6% of rams than with 1% in the flock. Rodríguez Iglesias, Ciccioi & Irazoqui (1997) did not obtain a higher percentage of ewes in estrus when they increased the percentage of rams from 8% to 16%.

Table 3. Effectiveness of rams from different breeds as teasers to induce estrus in anestrous ewes.

<b>Ram breed</b>		<b>Reference</b>
<b>More effective</b>	<b>Less effective</b>	
Dorset	Suffolk	Nugent, Notter & McClure, 1988
Dorset	Romney	Meyer, 1979 Knight & Lynch, 1980b Tervit & Peterson, 1978 Tervit, Havik & Smith, 1977 Knight, Dalton & Hight, 1980
Dorset	Romney X Finn	Meyer, 1979
Poll Dorset	Coopworth	Scott & Johnstone, 1994

### *Presence of ewes in estrus*

When rams are used as teasers, other social interactions are also involved, and in most published experiments it is impossible to discriminate which components are part of the ram effect. Ewes in estrus also influence reproductive activity in rams, mainly by an increase in LH pulses and testosterone levels during the first 4 to 8 hours of contact (Yarney & Sanford, 1983; González, 1989; González et al., 1989, 1991). Female-female effects have been demonstrated in Suffolk and Dorset ewes in close contact (Zarco et al., 1995), and suggested in Merino ewes (Oldham, 1980), similar to what occurs in cattle (Wright et al., 1994), goats (Restall, Restall & Walkden-Brown, 1995), and gilts (Prunier & Mounier, 1991). However, in some breeds (e.g., Romney) the presence of estrous ewes in itself does not induce ovulation in anestrous ewes (Knight, 1985).

The proportion of Romney ewes coming into estrus and ovulating increases when estrous ewes are introduced at the same time as rams ("social facilitation"; Knight, 1985; Muir, Smith & Wallace, 1989). A similar response was obtained when rams had been in contact with estrous ewes for a period before they were joined with ewes in anestrus (Knight, 1985). A higher percentage of anestrous Corriedale ewes ovulated when joined with estrous ewes at the same time as rams were introduced (Rodríguez Iglesias et al., 1991). Knight (1985) proposed that estrous ewes stimulate rams by increasing their testosterone levels (Knight, Ridland & Litherland, 1998) and therefore the rams become more effective in stimulating anestrous ewes. Rodríguez Iglesias et al. (1991) suggested that the continuous presence of estrous ewes is important because it provides visual cues to anestrous ewes of rams displaying sexual behavior. Rams that have been isolated from ewes and then are put together with ewes in estrus are more effective in stimulating anestrous ewes to ovulate than rams that have been in contact with ewes before the procedure takes place (Knight, Ridland & Litherland, 1998). Prior stimulation of rams by long dark periods (8L:16D) does not seem to further increase the number of ewes ovulating (González et al., 1986).

### *Other factors associated with rams*

Walkden-Brown, Restall & Henniawati (1993) found that bucks that had been at a high level of nutrition were more effective in stimulating anestrus does to ovulate than bucks at a low level of nutrition. However, in rams nutritional status does not influence serving capacity or the ability to induce ovulation (Fisher et al., 1994).

## **Factors associated with receptivity**

### *Depth of anestrus*

Seasonal anestrus is associated with a decrease in LH pulsatility (for reviews, see Martin, 1984; Gallegos-Sánchez, Malpoux & Thiéry, 1998) and with an absence of preovulatory surges of FSH and LH. The low LH pulsatility is due to two inhibitory mechanisms: (1) an increased negative feedback effect of estradiol on the hypothalamus, and (2) a direct effect of photoperiod on the hypothalamo-hypophyseal system controlling LH secretion (Goodman & Karsch, 1981).

Thomas and colleagues (1984) observed that some breeds are less sensitive to negative feedback of estradiol than others; ram effect stimulation had the result that more ewes from a less seasonal breed (Dorset) ovulated and conceived than did those from a more seasonal breed (Hampshire) (Nugent III, Notter & McClure, 1988). However, ewes from more seasonal breeds may not necessarily respond to the ram effect with an ovulation, even if they display an increase in their LH pulsatility (Minton et al., 1991).

There is little information about the physiological mechanisms that make a ewe respond to the ram effect. An indicator for anestrus depth may be LH pulse frequency, as it is higher in ewes that ovulate than in those not responding with an ovulation to the introduction of the rams (Martin et al., 1985). It has been reported that the percentage of ewes that respond to the ram effect is related to the percentage of ewes of the flock that ovulate spontaneously (Lindsay & Signoret, 1980). The response is also related to the period of the anestrus season: ewes are more receptive to the ram stimulus when rams are introduced close to the spontaneous onset of the breeding season (Cushwa et al., 1992; Oldham, Boyes & Lindsay, 1984).

### *Management: administration of melatonin*

Some investigators have administered melatonin – either by implants, with food, or by daily injections – to ewes in a shallow anestrus condition before introducing the rams. In most trials there was an increase in lambing rate (Crocker et al., 1992; Folch & Alabart, 1999; Kusakari & Ohara, 1996; Rekik, Bryant & Cunningham, 1991). In some of the trials, the response depended on the breed of the ewe (Gómez Brunet et al., 1995). An increase in the ewes coming in estrus (Kaya et al., 1998), an earlier conception (Crocker et al., 1992) or an increase in the conception rate (Gómez Brunet et al., 1995; Kusakari & Ohara, 1996) was observed in some breeds. However, the effect of melatonin treatment is probably not mediated by an increase in LH secretion (Gómez Brunet et al., 1995).

## **The ram effect in other phases of reproduction of the ewe**

The ram effect has also been used successfully to induce cyclic activity in prepubertal lambs and in postpartum ewes.

### *Puberty*

The introduction of rams to prepubertal lambs during the non-breeding season resulted in an increase of LH pulsatility, but ovulation occurred only when rams were introduced shortly before the onset of the breeding season (Al-Maully, Bryant & Cunningham, 1991; Hanrahan & O'Riordan, 1990). The percentage of 7- to 10-month-old lambs ovulating ranged between 30% and 60% according to breed and season (López, Alonso de Miguel & Gómez, 1985; Oldham & Gray, 1984). Dýrmondsson & Lees (1972) observed that the introduction of rams to lambs during the transition period from the non-breeding season to the breeding season did not affect the time of onset of mating activity but gave a better synchronization of receptivity. García & Pérez (1999) and Murtagh and coworkers (1984a) reported that the percentage of lambs that responded increased when lambs were preconditioned by previous exposure to rams.

### *Postpartum period*

There is little information about the use of the ram effect to induce estrus during the postpartum period. However, there are investigations showing that the interval from parturition to conception could be reduced when rams were introduced to postpartum ewes in autumn (Wright et al., 1989) and spring (Ungerfeld et al., 2001). Introduction of rams seems to have no effect on uterine involution of the ewe (Godfrey, Gray & Collins, 1998).

Postpartum Corriedale ewes and ewes that lambed several months earlier responded equally to the ram effect, with a similar number of ewes in estrus (Ungerfeld et al., 2001). However, in coincidence with Wright, Geytenbeek & Clarke (1990), the conception rate was lower in postpartum ewes, probably as a consequence of suckling and low body condition score.

The response of ewes to the ram effect during the postpartum period is time-dependent. In ewes that had lambed during the non-breeding season, Khaldi (1984) observed that the percentage of ewes that ovulated after the introduction of rams was higher at 75 days than at 15, 30, 45, or 60 days after parturition. We have compared the estrous response and the conception rate of suckling Corriedale ewes, and we did not observe significant differences in estrous response after introducing the rams at 5 (50.0%), 6 (42%), 7 (46%), or 8 (46%) weeks after parturition. However, conception rates in ewes stimulated 7 to 8 weeks after lambing were higher than in ewes stimulated at 5 to 6 weeks (37.5% vs. 5.9%,  $P < 0.05$ , L. Silva & R. Ungerfeld, unpublished results).

Cappai, Cognié & Branca (1984) reported that the response of Sarda ewes to the ram effect was related to the milk yield. A high milk yield reduced ovulation rate and delayed the LH surge. Prolactin concentrations – which are high in lactating ewes (Gómez Brunet & López Sebastian, 1991) – increase the negative feedback of estrogens on tonic LH secretion (Kann, Martinet & Schirar, 1976). However, similar to what has been observed in rebreeding of postpartum cattle (Williams & Ray, 1980), Poindron and colleagues (1980) observed that prolactin secretion was not related to the response of postpartum ewes to the ram effect.

## Outline and aims of the study

As discussed above, it is well accepted that the ram plays an important role in triggering reproductive cyclicality in anestrus ewes. The overall aim of the research presented in this thesis was to gain further knowledge of the reproductive response of Corriedale ewes exposed to the ram effect. A second aim was to develop easy and low-cost techniques for application in productive management for out-of-season estrus induction.

The ovarian response to the ram effect has previously been described through endocrine studies and laparoscopic observations (for review, see Martin et al., 1986). When rams are introduced, LH pulsatility is increased, and ovulation is induced in many of the ewes. However, this ovulation is not associated with heat. In some of the ewes, the first heat appears 17 to 20 days later, associated with the second ovulation, after a luteal phase of normal length. In others, there is an ovulation followed by a short luteal phase (4 to 5 days), then a second ovulation without signs of estrus, followed by a luteal phase of normal duration. Thereafter, another ovulation occurs associated with heat.

Socio-sexual effects on reproduction might be partially caused by stress mechanisms, because placing ewes together with animals from another flock induces a greater stress response than do, for example, spatial or visual isolation, confinement, or transportation (Baldock & Sibly, 1990).

The aims of the first study were (1) to characterize ovarian responses of anestrus Corriedale ewes to the ram effect using ultrasonography and to relate the ultrasonographic observations to serum concentrations of LH, FSH, and progesterone (**Paper I**); (2) to test whether the endocrine environment and follicular status of the ewes at the time of introduction of the rams will determine the ovarian response (**Unpublished results**); and (3) to test whether cortisol (and thus perhaps stress) is a component of the mechanism of the ram effect (**Unpublished results**).

In the first study, we could not determine whether there was a relationship between the growth status of the largest follicle present when rams were introduced and the ovarian response pattern. It has been reported that the growth status of the largest follicle present in the ovaries of cyclic ewes (Rubianes et al., 1997a) and anestrus ewes (Rubianes et al., 1997b) determines the ovarian response to a GnRH challenge. Estradiol-17 $\beta$  has been used to control follicular wave emergence in cows (for review, see Bo et al., 2002) and anestrus ewes (Meikle et al., 2001). In a pilot experiment performed before the second study (**Unpublished results**), we observed that estrus distribution changes if estradiol-17 $\beta$  is administered to anestrus ewes 3 or 5 days before the introduction of the rams. In the second study (**Paper II**), we manipulated follicular wave emergence with estradiol-17 $\beta$  to determine if there is a relationship between the growth status of the largest follicle and the following ovarian response pattern in anestrus ewes when rams are introduced.

The use of progestogen devices 12 to 14 days before the introduction of the rams to anestrus ewes ensures that heat is displayed coincident with the first ovulation followed by a luteal phase of normal duration. In previous trials, we have shown that short-term priming of anestrus ewes with intravaginal sponges impregnated with

medroxyprogesterone acetate (MAP) for 6 days followed by eCG administration is as effective as traditional long-term priming (12 days) for estrus induction (Ungerfeld and Rubianes, 1999a). We also showed that in combination with eCG, intravaginal sponges impregnated with 30 mg of MAP could be used with the same result as commercial sponges impregnated with 60 mg of MAP (Ungerfeld and Rubianes, 2002). In the third study (**Paper III**), the overall aim was to determine the effectiveness of different primings with MAP and the subsequent response to the ram effect. The objective of the first experiment was to measure the effectiveness of intravaginal sponges on estrous behavior and fertility when exposure to MAP was reduced from 14 to 10 and 6 days. The second experiment, using sponges with 20 mg, 40 mg, or 60 mg of MAP for 6 days, aimed to establish whether MAP dosage would affect estrous behavior and fertility. The objective of the third experiment was to elucidate the response, with regard to estrous behavior and fertility, to a single dose of MAP injected 0, 1, 3, or 5 days before introduction of rams into the flock.

Other commercial intravaginal devices containing other progestogens (progesterone, fluorogestone acetate) were not evaluated in **Paper III**. In particular, intravaginal devices containing progesterone, MAP, or fluorogestone acetate (FGA) have been used for estrus synchronization (Walker et al., 1989; Ungerfeld and Rubianes, 2002) with equal effectiveness. The aim of the fourth study (**Paper IV**) was to study the ram effect on the onset of estrus and the conception rate in Corriedale ewes after 6-day intravaginal primings with MAP, FGA, and progesterone in the non-breeding season.

In the fifth study (**Paper V**), the ovarian and endocrine responses of MAP-primed ewes to the ram effect during the breeding and the non-breeding seasons were investigated. Previously, we observed that MAP-primed cyclic ewes displayed an earlier and more synchronized estrus when submitted to the ram effect (Ungerfeld and Rubianes, 1999b). Thus, we wanted to characterize the ovarian response and the endocrine profiles in MAP-primed cyclic ewes stimulated by rams. In anestrus ewes (**Paper III and IV**), we observed that 30% to 50% of the primed ewes did not display heat until the second ovulation after a normal luteal phase (17 to 20 days later). A second objective of this study was to investigate how the endocrine status at ram introduction may affect estrous expression and first ovulation in anestrus ewes.

The introduction of estrous ewes together with the rams causes an increase in the percentage of anestrus ewes that ovulate (Rodríguez Iglesias et al., 1991). Yarney & Sanford (1983) and González and colleagues (1989, 1991) observed an increase of LH and testosterone concentrations in rams during the first 4 to 8 hours that the rams were in contact with estrous ewes. In the sixth study (**Paper VI**), the objectives were to determine if experienced adult rams and inexperienced young rams exhibit sustained changes in concentrations of LH, FSH, and testosterone, and in testicular consistency and size when such rams, together with estrous ewes, are introduced to anestrus ewes.



## Materials and methods

...es necesario contar historias del pueblo de tal forma que en vez de paralizarnos nos lleve a la acción.

Danny Glover

### Animals, locations, and general management

In all the experiments Corriedale ewes were used. Experiments were conducted in Uruguay at the Department of Physiology, Faculty of Veterinary Science, Montevideo (35° SL) (**Papers I and II**) and in 4 different commercial farms located near Colonia (35° SL) (Experiments 1 and 2 from **Paper III**, Experiment II from **Paper V, Paper VI**), near Trinidad (33° SL) (Experiment 1 from **Paper V**), near Baltasar Brum (31° SL) (**Paper IV**), and near Diego Lamas (31° SL) (Experiment 3 from **Paper III**).

Except for Experiment 1 from **Paper V**, all the experiments were performed during the non-breeding season. In all cases, ewes had lambed several months earlier, and lambs had been weaned at least 1 month before the introduction of the rams. In all of these experiments, rams were introduced during the first half of November. Experiment 1 from **Paper V** was conducted during the last days of April and the beginning of May.

In the field experiments, sheep grazed on native or improved pastures. In the lab experiments (**Papers I and II**), ewes received standard maintenance diets and water *ad libitum*. All ewes except the ewes in the control group in Experiment 1 (**Paper V**) were isolated from rams so that they could not see, hear, or smell each other (minimum distance = 1 km) for at least 1 month. It was reported that in the Corriedale breed, anestrous ewes that submit to the ram effect express maximum reproductive response when ewes in estrus are introduced together with the rams (Rodríguez Iglesias et al., 1991). Thus, in all the experiments estrous ewes were joined with the anestrous ewes at the same time as were the rams. The introduction of rams together with estrous ewes is considered as the ram effect throughout this thesis.

In the field experiments, rams were selected after a breeding soundness examination, which involved both a physical test of the ram's soundness and a test of reproductive soundness. Physical evaluation of feet and legs, body condition, vision, and any defect that might impair a ram's ability to breed was performed. The scrotum and testicles were measured and palpated, and the penis was physically examined. For the lab experiments performed in the Faculty (**Papers I and II**), rams were similarly examined, but we had to use the few rams we could obtain without selecting them. In order to minimize stress induced by change of location, rams were maintained near the city in conditions similar to those of the Department of Physiology for at least 3 weeks before they were joined with the ewes. However, the libido of the rams was not determined.

## Experimental designs

### *Paper I*

Eleven anestrus ewes were stimulated with the ram effect. Ovaries were scanned daily with transrectal ultrasound from 7 days before the rams were introduced until 18 days after ram introduction. Blood was collected once daily on Days 11, 7, 4, and 3, every 4 hours from ram introduction (Day 0) to 60 hours after ram introduction, twice daily from Days 3 to 8, and then once daily from Days 9 to 23. Blood samples were analyzed for FSH, LH, and progesterone concentrations.

### *Unpublished results related to the experiment of Paper I*

On Days 4 and 0, sampling took place every 15 minutes for 8 hours, beginning at 1200, and LH and FSH concentrations were measured in the samples. In addition to FSH, LH, and progesterone, testosterone and cortisol were assayed in the samples from the experiment of **Paper I**. Testosterone was measured as a substitute for estradiol-17 $\beta$ . Initially, samples were assayed for estradiol-17 $\beta$ , but concentrations were unusually high. As estradiol benzoate was used for inducing estrus in some ewes, we assume that the samples were contaminated. Scaramuzzi and coworkers (1980) suggested that testosterone might be a useful indicator of follicular estradiol production. Serum from 2 or 3 consecutive samples was pooled in order to obtain enough volume for the procedure.

### *A pilot study: response of anestrus ewes primed with estradiol-17 $\beta$ 3 or 5 days before the introduction of the rams (unpublished results).*

The experiment was performed in a farm located near Diego Lamas (31° SL) during November and December. One hundred seventy-eight nulliparous Corriedale ewes aged 2 years, weighing  $37.9 \pm 0.6$  kg, and with a body condition score (BCS) of  $3.3 \pm 0.1$  were used. Ewes were divided into 3 homogeneous groups according to BCS. Ewes received an intramuscular dose of 40 mg of estradiol-17 $\beta$  in 0.4 ml of corn oil on Day 5 (group Tr5, n = 56) or Day 3 (group Tr3, n = 61). Sixty-one ewes remained without treatment and served as a control group (group CU). On Day 0 (November 20), all ewes were placed together with 15 sexually experienced Corriedale rams with markers and 20 additional ewes in estrus. Sexual receptivity was estimated from marks on the rumps of the ewes twice daily from Days 0 to 5 and Days 16 to 27, and once daily on Days 8, 11, and 14. All ewes were managed together until marked by the rams. Marked ewes were taken out from the flock with rams so as to maintain the ram:ewe ratio. Onset of estrus was considered to be at the time between the last control in which an ewe was unmarked and when it was detected marked by a ram. Pregnancy was determined by transrectal ultrasonography on Day 54.

## *Paper II*

Thirteen anestrous ewes were divided in 2 experimental groups and stimulated with the ram effect. Seven ewes received a single dose of estradiol-17 $\beta$  5 days before rams were introduced, and 6 ewes remained as untreated controls. Ovaries were scanned daily with transrectal ultrasound from 9 days before rams were introduced for 24 days. Blood samples were obtained, and FSH, LH, estradiol-17 $\beta$ , and progesterone concentrations were measured.

## *Paper III*

### Experiment 1

One hundred ninety-eight multiparous ewes were used. Ewes were divided into 4 groups. Intravaginal sponges containing 60 mg of MAP were inserted on Day 14 (n = 43), Day 10 (n = 48) and Day 6 (n = 48). Fifty-nine untreated ewes served as a control group. At sponge withdrawal (November 1) all ewes were placed together with 14 sexually experienced Corriedale rams with markers and 36 ewes in estrus. Sexual receptivity was estimated from marks on the rumps of the ewes twice daily from Day 0 to Day 5 and again from Day 17 to Day 28, and once daily on Days 8, 11, and 14. All ewes were managed together until marked by the rams. Marked ewes were taken out of the flock and rams were removed as necessary to maintain the ram:ewe ratio. Onset of estrus was considered to be between the last control at which an ewe was unmarked and the time when marking by a ram was first observed. To determine pregnancy status, transrectal ultrasonography was performed on Day 40 in ewes that were mated on Days 1 through 5. A second ultrasonographic examination was performed on Day 60 in ewes that were first mated after Day 17.

### Experiment 2

Two hundred seven multiparous ewes were used. On Day 6, intravaginal sponges containing either 20 mg (n = 46), 40 mg (n = 47), or 60 mg of MAP (n = 48) were inserted. Sixty-six untreated ewes served as controls. Sponges remained *in situ* for 6 days. At sponge withdrawal (November 14), all ewes were placed with 17 sexually experienced marking Corriedale rams and 50 additional ewes in estrus. Sexual receptivity was determined twice daily from Day 0 to Day 7 and from Day 17 to Day 25, and every second day from Day 8 to Day 16. Ewes were managed as in Experiment 1. Pregnancy was determined on Day 36 in ewes that were mated between Day 1 and Day 7. Blood was collected for progesterone determination each 24 to 48 hours from Day 0 to the onset of estrus.

### Experiment 3

One hundred ninety-one nulliparous 2-year-old ewes were used for this experiment. Ewes were divided into 5 groups. Ewes were treated with 2.5 mg of MAP injected i.m., on Day -5 (n = 40), Day -3 (n = 38), Day -1 (n = 38) or Day 0 (n = 37). Thirty-eight untreated ewes served as a control group. On Day 0 (November 20), all ewes were placed with 11 sexually experienced Corriedale marking rams and 20 additional ewes brought into estrus between Day 0 and Day 2. Sexual receptivity was estimated once daily from Day 0 to Day 5 and from Day 16 to Day 28, and once on Days 8, 11, and 14. The flock was managed as in Experiment 1 and pregnancy was determined by ultrasonography on Day 58.

### *Paper IV*

One hundred eighty-nine Corriedale ewes were used in the experiment. Ewes were isolated (by sight, sound, and smell) from rams (minimum distance = 4000 m) for more than 7 months. Ewes were divided into 4 groups. On Day -6 (Day 0 = introduction of the rams), MAP sponges (60 mg, n = 49), FGA sponges (30 mg, n = 49), or devices containing progesterone (CIDR, 0.3 g, n = 46) were inserted. Forty-five ewes served as a control group. The sponges remained *in situ* for 6 days. At sponge withdrawal (Day 0), all ewes were placed with sexually experienced Corriedale rams with markers for estrous detection and 50 additional ewes in estrus. All ewes were managed together until marked by rams. Marked ewes were removed from the flock with rams to maintain the ram:ewe ratio. Estrous ewes were identified twice daily from Days 0 to 6 and 17 to 25, and once daily from Days 7 to 16. The onset of estrus was considered to occur at a point halfway between the last control when the ewe was not marked by a ram and the first one in which it was. To determine pregnancy status, transrectal ultrasonography was performed 5 to 6 weeks after estrus.

### *Paper V*

#### Experiment 1

Altogether 71 multiparous Corriedale ewes were used in the mid-breeding season. On Day -34 (Day 0 = ram introduction), the experimental ewes were divided into two groups: a ram effect group (n= 36) and a control group (n=35). Ewes in the ram effect group were isolated from rams so that they could not see, hear, or smell them (minimum distance = 1 km). Ewes in the control group remained close to the pen where the rams were kept. Intravaginal sponges of 60 mg MAP were inserted in ewes of both groups on Day -12. At sponge withdrawal, all the ewes were mixed and placed in the same paddock with 8 sexually experienced adult Corriedale rams with markers. Ewes in estrus were identified at 12-hour intervals, from 12 to 96 hours after introduction of the rams. At 4 to 6 days after estrus, ovulation and the ovulation rate were assessed by mid-ventral laparoscopy performed under local anesthesia. To determine pregnancy status, transrectal ultrasonography was performed 4 weeks after estrus.

A detailed study of the ovarian and endocrine patterns of the follicular phase was conducted in 8 ewes from each group. Daily ultrasonographic observations of ovaries were performed by the same operator in all ewes from Hour -72 (Hour 0 = introduction of the rams) to Hour 0, and at 12-hour intervals either until ovulation had occurred or until Hour 96. Before each ultrasonographic examination, blood samples were collected and assayed for FSH, LH, and estradiol-17 $\beta$ .

#### Experiment 2

Fourteen adult multiparous Corriedale ewes were used. From Day -30 (Day 0 = day on which rams were introduced), ewes were isolated from rams in terms of sight, sound, and smell (minimum distance = 1 km). On Day -6, intravaginal sponges containing 60 mg of MAP were inserted in all ewes. At sponge withdrawal, ewes were placed together with 3 adult, sexually experienced Corriedale rams with markers and 10 ewes in estrus. The anestrous ewes were checked twice daily from Day 0 to Day 5 for onset of estrus. Transrectal ultrasonographic examinations of ovaries were performed every 12 hours, from Hour -96 until ovulation occurred or until Hour 120. Blood was collected from all animals on Days -12 and -8. On Day -8, all animals were fitted with jugular vein catheters, which

were used to collect blood samples until Day 7. On Day 6, before the sponges were inserted, sampling took place every 15 minutes for 8 hours in 12 animals. From Day -4 to Day 0, samples were obtained every 12 hours, and from Day 0 until Hour 120, samples were obtained every 4 hours. A single sample was obtained on Days 8, 11, and 14. Samples taken until Day 5 were used for measurement of FSH, LH, and estradiol-17 $\beta$ ; samples taken from Day 5 to Day 14 were measured for progesterone.

### *Paper VI*

Eight experienced adult (4 to 6 years) and 8 inexperienced yearling (1 to 1.5 years) Corriedale rams were used in the experiment. Rams were isolated from ewes (minimum distance = 1 km) for 30 days. Approximately 200 ewes were managed as in Experiment 1 from **Paper III**. Briefly, 100 of the ewes were primed for 6 to 14 days with intravaginal sponges containing MAP (60 mg) in order to ensure that a significant number of ewes were in estrus during the first days of contact with rams. An additional 36 ewes were brought into estrus and introduced to the anestrous ewes together with the rams. Ewes were checked twice daily from Days 0 to 5 and 16 to 20, and once on Days 8, 11, and 14 for onset of estrus. On Days 1 to 4, 111 ewes came into estrus, and 93 came into estrus on Days 17 to 20. No ewes came into estrus between Day 5 and Day 16. Rams remained in contact with all of the ewes until Day 20.

Changes in LH, FSH, and testosterone concentrations, testicular firmness and resilience, and scrotal circumference were monitored for 20 days, at which time rams were used to stimulate the MAP-primed anestrous ewes. Testicular consistency was evaluated for firmness (amount that the tissue can be pressed) and resilience (springiness, amount that the tissue springs back when pressed). Testicular firmness and resilience were evaluated according to the technique described by Galloway (1998). The same person performed all measurements.

## **Comments on methods**

### *Ultrasound*

The ultrasound technique for scanning and determination of ovarian structures has been validated in sheep by Viñoles, Meikle & Forsberg (2003). In **Papers I and V** a Pie Medical 480 (Maastricht, the Netherlands) equipment with a dual linear-array probe (5/7.5 MHz) was used. In **Paper II** an Aloka 500 (Tokyo, Japan) provided with a 7.5 MHz linear-array probe was used. During ultrasound examination, sketches of ovaries were made to record the diameter and position of follicles >2 mm in diameter. The observations were also recorded on video using individual videocassettes for each ewe in order to verify the sketches with real-time data.

### *Estrous behavior and pregnancy diagnosis*

Estrous behavior was always recorded with marker rams. Pregnancy diagnosis was done with the same ultrasound equipment used for ovarian sketching.

### *Hormone measurements*

Concentrations of FSH, LH, estradiol-17 $\beta$ , progesterone, testosterone, and cortisol were measured by radioimmunoassay (RIA). The blood sampling procedures, the serum management, and the RIAs for FSH, LH, estradiol-17 $\beta$  and progesterone are described in the papers (**I, II, IV, V, and VI**).

Testosterone was measured with a solid-phase RIA kit (Count-A-Count TKPG, Diagnostic Products Corporation, Los Angeles, CA, USA). According to the manufacturer, the antiserum shows very little cross-reaction with other steroids (estradiol-17 $\beta$  = 0.02%). Several dilutions of ovine serum produced displacement curves parallel to the standard curve. The intra-assay coefficient of variation was 8.3%, and the inter-assay coefficient of variation was 15.5%. The detection limit of the assay was 50 pmol/L. Results are expressed in pmol/L.

Cortisol concentrations were determined with a direct solid-phase <sup>125</sup>I RIA method (Count-A-Count TKPG, Diagnostic Products Corporation, Los Angeles, CA, USA) previously validated for ovine serum (Van Lier, 1998). According to the manufacturer, the antiserum exhibits low cross-reactivity with other steroids. The detection limit of the assay was 5 nmol/L. The intra-assay and inter-assay coefficients of variation were 4.6% and 8.5%, respectively. Results are expressed in nmol/L.

### *Definitions*

#### Paper I

Luteal activity was defined as the presence of progesterone concentrations above 1.6 nmol/L in 3 or more consecutive samples (for a duration of >36 hours). A normal luteal phase (NLP) was defined as the presence of progesterone concentrations above 1.6 nmol/L for at least 10 days.

An LH surge was defined as being at least six times higher than basal concentrations. LH pulses were defined using the following criteria (slightly modified from Van Lier, 1998): (1) a pulse had to occur within 3 samples of the previous nadir, with a rise of more than 0.5 mg/L from the previous sample, or if the rise occurred after the third sample from the nadir, the rise had to be 1 mg/L or more, and (2) the subsequent decline to the next nadir had to be more than 1 mg/L. LH pulses were counted for each animal during each period. LH basal levels were calculated after the values contributing to pulses were removed.

#### Unpublished results related to the experiment of **Paper I**

LH pulses were defined using the following criteria (slightly modified from Van Lier, 1998): (1) a pulse had to occur within 3 samples of the previous nadir, with a rise of more than 0.5  $\mu$ g/L from the previous sample, or if the rise occurred after the third sample from the nadir, the rise had to be 1  $\mu$ g/L or more, and (2) the subsequent decline to the next nadir had to be more than 1  $\mu$ g/L. LH pulses were counted for each animal during each period. LH basal levels were calculated after the values contributing to pulses were removed.

#### Paper II

Luteal activity was defined as the presence of progesterone concentrations above 1.6 nmol/L in 4 or more consecutive samples when concentrations were measured every 12 hours, or above 0.9 nmol/L in at least 5 consecutive samples when values were measured every 4 hours. An NLP was defined as the presence of luteal activity for at least 10 days; a short luteal phase (SLP) was defined as luteal activity for no more than 4 days.

An LH surge was defined as being at least 8 times higher than the basal levels (mean value of all samples).

#### Paper III

The existence of luteal phases was established according to the following criteria: (1) progesterone concentration exceeded 1.6 nmol/L in 2 consecutive samples taken 48 hours apart; (2) progesterone concentration exceeded 3 nmol/L in one sample when the times to the previous sample and to the next sample were both at least 48 hours. An NLP was defined as a phase with luteal levels of progesterone for at least 10 days, and exceeding 6 nmol/L in at least one sample. An SLP was defined as a luteal phase with luteal levels of progesterone for at least 48 hours and no longer than 4 days.

#### Paper V

Luteal activity was defined as the presence of progesterone concentrations > 1.6 nmol/L in 3 or more consecutive samples. LH pulse frequency during the intensive bleeding period was defined using the following criteria: (1) pulse values had to be higher than the mean value + 2 standard deviations, and (2) the subsequent decline to the next nadir had to be > 1 µg/L. An LH surge was defined as being at least 6 times the value of basal levels.

#### *Statistical analyses*

All the statistical analyses in this thesis were performed with SAS.

#### Paper I and unpublished results related to the experiment of Paper I

Concentrations of FSH were compared using an ANOVA for repeated measures after log transformation; time points were compared using least significant difference (LSD). Testosterone and cortisol concentrations were compared using ANOVA for repeated measures after log transformation. The frequency of LH pulses and the follicular populations were compared with Mann-Whitney test.

#### Paper II

The size of the largest follicle and days of follicular wave emergence were compared with ANOVA, variances were compared with Bartlett's test, and frequencies were compared with a chi-square test. Hormonal profiles were analyzed with a general linear model procedure using ANOVA for repeated measures after log transformation. In the Pilot study, frequency of ewes in estrus was compared with chi-square test; estrus distribution between the 3 groups was compared with Kruskal-Wallis test; individual groups were compared with Mann-Whitney test.

#### Paper III

Frequencies of ewes in estrus and pregnant ewes were compared by a chi-square test; individual groups were compared by Fisher's exact probability test. The times from the introduction of rams to the onset of estrus, and to the onset of luteal activity were each compared by ANOVA.

#### Paper IV

Frequencies of ewes in estrus and rates of conception were compared by a chi-square test. The interval from withdrawal of the intravaginal device to onset of estrus was compared by ANOVA.

#### Paper V

Mean intervals from sponge withdrawal to estrus were compared by ANOVA; frequencies of ewes in heat were compared by Fisher's exact probability test. Ovulation rate was compared by the Kruskal-Wallis test (Experiment 1). LH pulse frequency and follicular populations were compared by the Kruskal-Wallis test; the diameter of the largest follicle was compared by ANOVA; and LH surge values were compared by ANOVA after log transformation (Experiment 2). Hormonal profiles and the growth profiles of follicles were analyzed with a general linear model procedure using an ANOVA for repeated measures. Hormonal data were analyzed after normalization by log transformation.

#### Paper VI

Hormone concentrations, testicular firmness and resilience, and scrotal circumferences were analyzed after log transformation for normalization using a mixed procedure. The statistical model included the effects of group (experienced adult and inexperienced young rams), day, and the interaction between group and time, as well as the random effect of ram within group.



## Results

Es más fácil desintegrar un átomo que un pre-concepto

Albert Einstein

### **Ovarian responses of anestrus ewes to the ram effect (Paper I)**

Using ultrasound scanning we observed a wide range of ovarian responses. Only 1 ewe had a classical response, with an LH surge and ovulation of the largest follicle 2 days after the introduction of the rams, followed by an SLP. There was a second LH surge and ovulation followed by an NLP.

We also observed some other ovarian patterns:

Three ewes had delayed ovulations (Days 5 to 7) without heat followed by NLPs. Two ewes had SLPs that were not preceded by ovulation. In one of those ewes an LH surge was detected. Another ewe had an NLP preceded by an LH surge but without ovulation. Four ewes did not ovulate and had no luteal phases. One of these ewes had an LH surge 24 hours after rams were introduced.

All the ewes that showed ovarian responses (luteal phases) after the introduction of the rams returned again to an anestrus condition without displaying estrous behavior during the experimental period (until Day 25) and did not show another luteal phase after the initial described response.

In ewes with an LH surge ( $n = 7$ ), serum FSH concentrations increased concurrently with the LH surge, and remained high during the next 24 hours. Thereafter, FSH concentrations decreased and remained stable for the rest of the sampling period.

### **Anestrus depth and the ovarian and endocrine responses to the ram effect (unpublished results related to the experiment of Paper I)**

Ewes were divided into 2 groups dependent on the response to the ram effect: ewes presenting luteal phases ( $n = 7$ ; LP) and ewes not presenting luteal phases ( $n = 4$ ; NP). There were no significant differences between groups in the number of large follicles and the diameter of the largest follicle before introducing the rams (Days  $-7$  to  $0$ ) and during the first 4 days after the introduction of the rams (Table 4). Independently of the group, the number of large follicles and the diameter of the largest follicle were bigger or tended to be bigger during the first 3 days after introduction of the rams than during the period before ram introduction. From Day 4 and onwards, no significant differences were observed in any of the variables studied.

Table 4. Ovarian and endocrine responses of ewes to the ram effect. Ewes were classified as presenting luteal phases (LP, n = 7) or not presenting (NP, n = 4) (see details in the text). Data are expressed as mean  $\pm$  SEM.

	LP	NP	P
Number of follicles $\geq$ 4 mm			
Before rams	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	Ns
Day 1	1.1 $\pm$ 0.1 <sup>***</sup>	1.0 $\pm$ 0.0 <sup>***</sup>	Ns
Day 2	1.0 $\pm$ 0.3 <sup>*</sup>	0.8 $\pm$ 0.3 <sup>**</sup>	Ns
Day 3	1.0 $\pm$ 0.3 <sup>*</sup>	1.0 $\pm$ 0.0 <sup>*</sup>	Ns
Day 4	0.7 $\pm$ 0.3	0.5 $\pm$ 0.3	Ns
Diameter of the largest follicle (mm)			
Before rams	3.7 $\pm$ 0.1	3.7 $\pm$ 0.1	Ns
Day 1	4.6 $\pm$ 0.3 <sup>***</sup>	4.5 $\pm$ 0.2 <sup>***</sup>	Ns
Day 2	4.1 $\pm$ 0.1 <sup>**</sup>	4.5 $\pm$ 0.3 <sup>***</sup>	Ns
Day 3	4.9 $\pm$ 0.3 <sup>***</sup>	4.4 $\pm$ 0.2 <sup>***</sup>	Ns
Day 4	3.9 $\pm$ 0.2	3.8 $\pm$ 0.7	Ns
LH pulses (8 h)			
Before rams	1.2 $\pm$ 0.3	0.0 $\pm$ 0.0	< 0.02
After rams	3.1 $\pm$ 0.9 <sup>*</sup>	2.0 $\pm$ 0.7 <sup>**</sup>	Ns
FSH levels ( $\mu$ g/L)			
Before rams	16.7 $\pm$ 0.5	13.9 $\pm$ 0.5	< 0.01
After rams	18.4 $\pm$ 0.6	13.7 $\pm$ 0.5	< 0.01

Asterisks indicate significant differences for each value with respect to values before rams: \* ( $P < 0.1$ ); \*\* ( $P < 0.05$ ); \*\*\* ( $P < 0.01$ ).

The last column indicates  $P$  values for the same row (LP vs. NP).

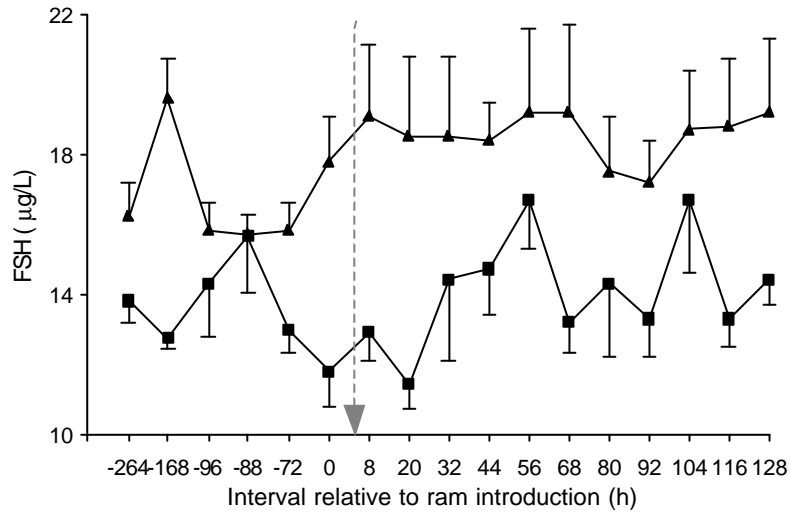


Figure 4. Concentrations of FSH relative to ram introduction (arrow). Ewes were classified as presenting (- $\blacktriangle$ -) or not presenting (- $\blacksquare$ -) luteal phases (see details in the text). Unpublished results.

Ewes from the LP group had significantly more LH pulses over an 8-hour period before the introduction of the rams (Table 4) and higher FSH levels before and after the introduction of

the rams (Table 4; Figure 4). Figure 5 shows LH concentrations in 1 LP ewe during 8-hour periods 4 days before and immediately after the introduction of the rams.

### **Testosterone and cortisol concentrations in the response to the ram effect (unpublished results related to the experiment of Paper I)**

Testosterone concentration did not vary significantly over time. However, when the values were normalized with respect to the LH surge (6 LP ewes and 1 NP ewe) (Figure 6), an increase was observed at the time of the LH surge.

Individual cortisol concentrations ranged between 9.6 and 44.8 nmol/L without significant differences before and after rams were introduced.

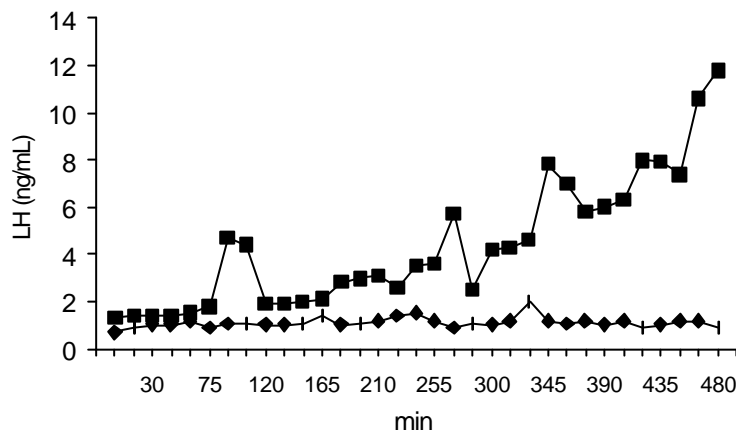


Figure 5. LH concentrations in 1 LP ewe during 8-hour periods 4 days before (-♦-) or immediately after the introduction of the rams (-■-). Unpublished results.

### **A pilot study: response of anestrus ewes primed with estradiol-17b 3 or 5 days before the introduction of the rams (unpublished results)**

All ewes that came into estrus did so after Day 16. The total number of ewes in estrus was similar between groups (57/61, 53/61 and 46/56 for groups CU, Tr3, and Tr5, respectively). Estrous onset was on Days  $22.0 \pm 0.3$ ,  $20.9 \pm 0.4$  and  $22.5 \pm 0.4$  for groups CU, Tr3, and Tr5, respectively. Estrus distribution (Figure 7) was different between the three groups ( $P < 0.001$ ); CU differed from groups Tr5 ( $P = 0.01$ ) and Tr3 ( $P < 0.05$ ); Tr3 differed from group Tr5 ( $P < 0.0001$ ).

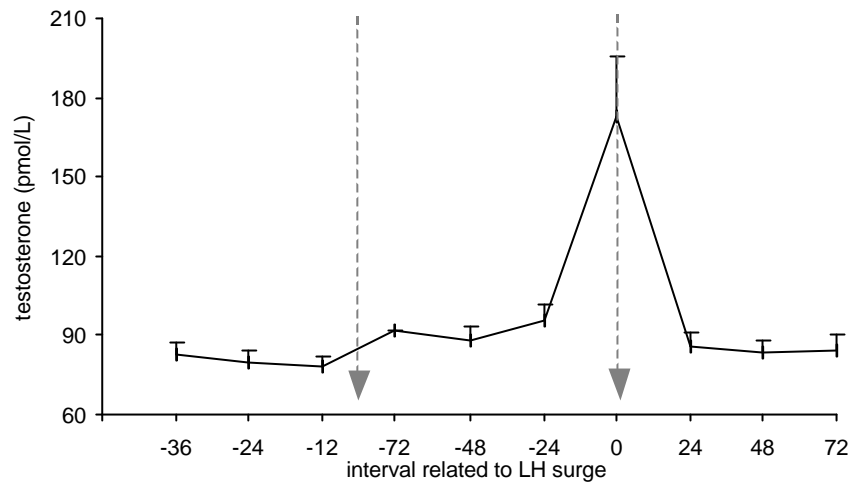


Figure 6. Testosterone concentrations from 7 ewes before and after the introduction of the rams (first arrow), normalized with respect to the LH surge (second arrow). In 2 ewes that elicited 2 LH surges, concentrations were normalized with respect the first surge. Unpublished results.

### **Response of anestrus ewes to the ram effect after follicular wave synchronization with a single dose of estradiol-17 $\beta$ (Paper II)**

Treatment with estradiol-17 $\beta$  induced rapid LH and FSH surges. The emergence of the following follicular wave after estradiol-17 $\beta$  treatment was synchronized in estradiol-17 $\beta$  treated-ewes compared with untreated ewes.

The growth status of the largest follicle at the moment of ram introduction was unrelated to the ovarian response pattern. When rams were introduced, 7/7 treated ewes had the largest follicle in a growing stage as compared with 3/6 control ewes. Five control and 4 treated ewes ovulated after the introduction of the rams. Only 1 ewe from each group developed an NLP; 4 control and 3 treated ewes had SLPs. Three ewes that did not ovulate (1 control and 2 treated) had SLPs originating from non-ovulatory follicles. After the first luteal phase, all ewes returned to anestrus without a second ovulation or luteal phase. One treated ewe did not ovulate or show any changes in progesterone serum concentrations.

### **Medroxyprogesterone priming and response to the ram effect in Corriedale ewes during the non-breeding season (Paper III)**

We observed the following:

Short-term priming (6 days) with MAP was as effective as traditional long-term priming (14 days) in terms of signs of estrus, estrus distribution, and fertility (Experiment 1).

Intravaginal sponges impregnated with either 20 or 40 mg of MAP were as effective as commercial sponges (60 mg) when used in a 6-day priming. The percentage of ewes in

estrus, estrus distribution, and conception rates were similar between treatments (Experiment 2).

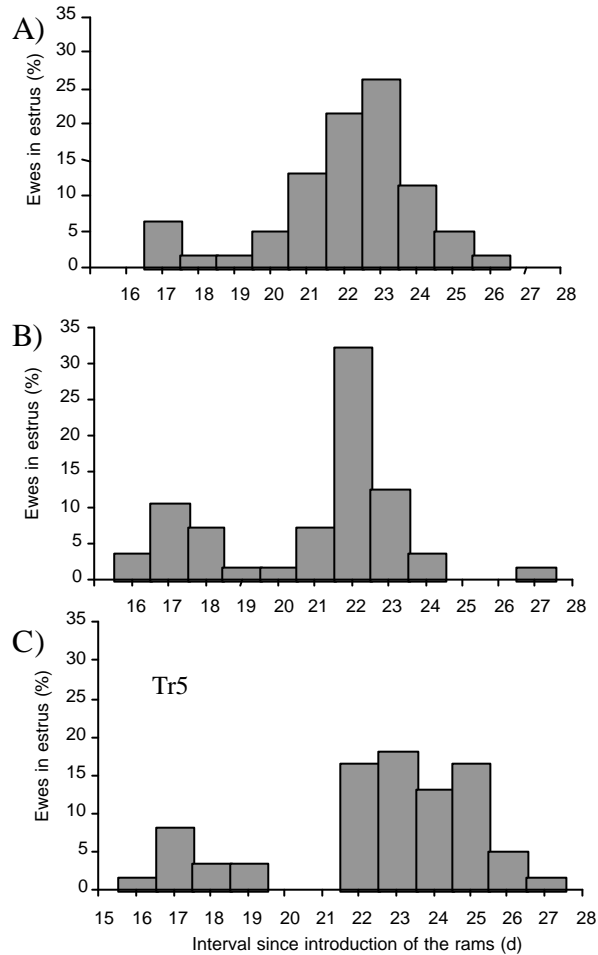


Figure 7. Estrus distribution of anestrous Corriedale ewes stimulated with the ram effect. Ewes remained untreated (A), or received 40 mg of estradiol-17 $\beta$  3 days (B), or 5 days (C) before rams were introduced. Unpublished results.

In the primed groups, 50% to 70% of the ewes that came into estrus did so during the first 3 to 4 days after the introduction of the rams (Experiments 1 and 2).

A single MAP dose administered 3 to 5 days before the introduction of the rams improved the synchronization of the induced estrus approximately 17 to 18 days after the introduction of the rams (Experiment 3).

In ewes that responded to the ram effect with luteal phases of normal length, the day of onset of the luteal phase significantly influenced the length of the luteal phase (Figure 8).

In primed ewes, the conception rate was lower in the first estrous period (Days 1 to 5) compared with second estrous period (Days 17 to 28) (Experiment 1).

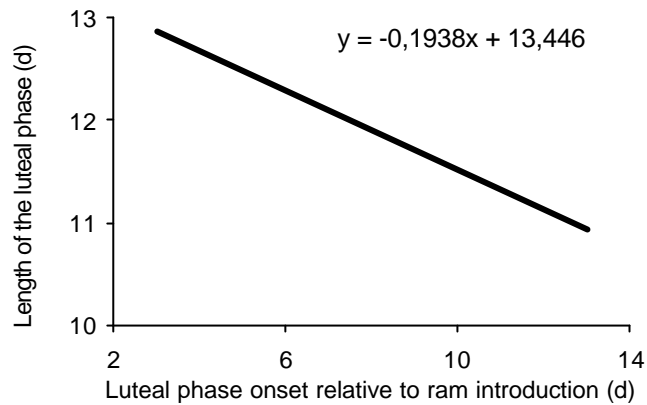


Figure 8. Influence of the day of onset of normal luteal phases on the length of the luteal phase.

#### **Response of Corriedale ewes to the ram effect after priming with medroxyprogesterone, fluorogestone, or progesterone in the non-breeding season (Paper IV)**

We determined that commercial intravaginal devices containing progesterone, MAP, or FGA could be used in 6-day primings with similar results. Signs of estrus, estrus distribution, and conception rates were similar in ewes primed with the 3 different devices. Progestogen priming increased the number of ewes in estrus compared with unprimed ewes.

The trend in fertility from the first to estrous period to the second was opposite to that obtained in Experiment 2 from **Paper III**. In **Paper IV** a higher conception rate was observed in ewes coming in heat during the first period (Days 0 to 3) than in the estrous period observed after Day 17.

### **Endocrine and ovarian changes in response to the ram effect in medroxyprogesterone-primed Corriedale ewes during the breeding and non-breeding seasons (Paper V)**

We did not observe any differences in endocrine status (FSH, LH, or estradiol-17 $\beta$  concentrations), growth profile of the ovulatory follicle, ovulation rate, estrus distribution, or pregnancy rate induced by the ram effect in MAP-primed cyclic ewes or control ewes at the mid-breeding season (Experiment 1).

In Experiment 2, we observed that anestrous ewes that responded to the ram effect with an ovulation and heat had higher FSH and estradiol-17 $\beta$  concentrations before the introduction of the rams than did ewes that had an ovulation without expressing heat or that did not ovulate. All ewes that ovulated had an LH surge and reached higher maximum FSH levels than ewes that did not ovulate, of which none had an LH surge. The endocrine pattern of the induced follicular phase of ewes that came into estrus was similar to a normal follicular phase. In ewes that ovulated without signs of estrus, we did not observe an increase in estradiol-17 $\beta$  concentrations or a simultaneous decrease in FSH levels. There were no differences in growth profiles of the largest follicle between ewes that displayed estrus and ovulated, those that ovulated without heat, and those that did not ovulate.

### **The ewe effect: endocrine and testicular changes in experienced adult and inexperienced young Corriedale rams used for the ram effect (Paper VI)**

Increases were observed in LH and testosterone concentrations and in testicular firmness and resilience during the first 4 days when rams were in permanent contact with estrous ewes. During the following 13 days, when rams were in contact with non-estrous ewes, LH and testosterone concentrations decreased. When ewes exhibited estrus 17 to 20 days after ram introduction, concentrations of testosterone increased. Testicular firmness and resilience remained high throughout the period.

Adult experienced rams had significantly higher FSH concentration than inexperienced young rams. The opposite was observed in LH levels, which were higher in the inexperienced rams. Testosterone concentrations were similar for both categories of rams. Testicular firmness, testicular resilience, and scrotal circumference were also higher in experienced adult rams than in inexperienced young rams throughout the observational period.

## Discussion

Quien altere los cuentos de hadas para calzarles sus propias opiniones, cualesquiera sean, es culpable de un acto de soberbia y se apropia de lo que no le pertenece.

Charles Dickens

### Ovarian response patterns to the ram effect

We observed a wide range of ovarian responses to the ram effect. Many of them differ from the previously reported typical response of ovulation (relatively soon after ram exposure) followed by an SLP or NLP (Martin et al., 1986). First of all, we confirmed the suggestion of Fulkerson, Adams & Gherardi (1981) about the existence of delayed ovulations 6 to 9 days after the introduction of rams (**Papers I and II**). Cushwa and colleagues (1992) have also described this response through laparoscopic observations.

Other responses, such as the existence of short luteal phases caused by anovulatory follicles (**Paper I**) have been described in prepubertal lambs (Ryan & Foster, 1978; Berardinelli et al., 1980) and at the beginning of the breeding season in adult ewes (Ravindra & Rawlings, 1997). The quick return to anestrus of those ewes is in accordance with observations from Atkinson & Williamson (1985). Luteinized follicles have been reported as a response to the ram effect (Knight, Tervit & Fairclough, 1981), and were also observed during the transition to the breeding season (Bartlewski et al., 1998), and after the administration of GnRH to anestrus ewes (Rubianes et al., 1997b).

This confirms the usefulness of ultrasonographic studies accompanied by hormonal measurements as a powerful technique to characterize ovarian status. In previous experiments, some of these response patterns might have been masked because of the techniques used. It should be considered that the classical response pattern described by Martin and coworkers (1986) was described mainly from experiments performed in Merino ewes, one of the less seasonal breeds. Only in a small number of experiments performed with ewes from other breeds have the ovaries been scanned by ultrasound. The factors that determine the different ovarian response patterns still remain unknown. The presence of a growing follicle when rams are introduced does not guarantee the prevalence of NLPs, as happens when anestrus ewes are subjected to pharmacological treatments (Rubianes et al., 1997a, 1997b) that do not need the physiological response of the hypothalamo-pituitary axis of the ewe. However, as in the **Pilot study** performed before Paper II ewes treated with estradiol-17 $\beta$  came more synchronized into estrus, more research should be performed before obtaining a definitive conclusion.

### The endocrine response of the ewe to the ram effect

**In the unpublished study related to Paper I** and in Experiment 2 of **Paper V**, an increase in FSH concentration was registered at the time of the LH surge, similar to that reported by Poindron and colleagues. (1980). Atkinson & Williamson (1985) observed a



quick fall in FSH levels in response to the introduction of rams, and levels remained low for at least 10 days. One explanation for the different results could be that Atkinson & Williamson (1985) analyzed FSH levels in relation to the introduction of the rams and not to the LH surge, so the different times needed by different ewes to elicit an LH surge could have masked the changes. In all ewes that showed an LH surge, a testosterone peak was registered at the same time. A testosterone peak coinciding with the LH surge during the follicular phase of cyclic ewes was previously reported by Baird, Swanston & McNeilly (1981). Our results confirm and extend that information to the follicular phase induced by ram stimulation in anestrus ewes, suggesting also an increase in estradiol concentrations.

Cortisol levels did not present important variations during the experimental period. Moreover, concentrations were similar to basal levels observed by Van Lier (1998) using ewes from the same breed and the same technique to measure cortisol, suggesting that stress is not a component of the ram effect.

## **Factors associated with receptivity of the ewe to the ram effect**

### *Anestrus depth*

The increase in LH pulsatility observed in **the unpublished study related to Paper I** coincided with an increase in the number of large follicles and with an increase in the diameter of the largest follicle. This should be expected because terminal preovulatory follicular growth is dependent on LH pulsatility (Baird & McNeilly, 1981). Atkinson & Williamson (1985) reported an increase in the number of large follicles, after the introduction of rams to anestrus ewes, coincident with a quick increase in LH pulsatility. We observed that ewes that responded with a luteal phase and those not responding had similar increases in the diameter of the largest follicle, coincident with an increase in LH pulsatility. This suggests that the response of an ewe to the ram effect with a luteal phase is unrelated to the maximum diameter attained by the largest follicle present after rams are introduced. Our results, similar to those observed after administration of PGF-2 $\alpha$  and GnRH to cyclic ewes (Rubianes et al., 1997a), lead us to conclude that the endocrine environment to which the follicle is exposed during its development will determine whether it will respond to the ram stimulus with a luteal phase or not.

Although more work has to be performed to determine what anestrus depth means, our results are in agreement with previous observations (Martin et al., 1985). The LH pulsatility has been proposed as one parameter to estimate anestrus depth (Martin et al., 1985). We also found that FSH concentrations differed between ewes responding to the introduction of the rams with a luteal phase or not. In **Paper V** we also observed that estradiol-17 $\beta$  concentrations might be related to estrous behaviour in progestogen-primed ewes stimulated with the ram effect. However, specific values from any hormone that may be used to characterize individual anestrus depth should be considered only with data from a specific flock, because different basal hormone concentrations may be related to breed, the anestrus period, nutritional status of the animals, etc.

Overall, the reproductive response of a ewe to the ram effect is related to anestrus depth. The response may also be influenced by the strength of the stimulus (e.g., libido of the rams, presence of other ewes in estrus).

## **Factors associated with the stimulus of the ram effect**

### *Presence of ewes in estrus*

In **Paper VI**, we observed that rams responded to the introduction of estrous ewes with fast increases in LH and testosterone levels and that the concentrations of both hormones remained high for several days. LH and testosterone levels decreased when ewes in contact with the rams were not in estrus (after Day 5). This suggests that the endocrine system of the rams responded to estrous ewes. The number of ewes that ovulate increases with sexual performance (Perkins & Fitzgerald, 1994) and high testosterone levels of rams (Rosa, Juniper & Bryant, 2000). Thus, the high number of anestrus ewes that ovulated when estrous ewes were present (Rodríguez Iglesias et al., 1991) in our experiments may partially be explained by increasing testosterone levels of the rams. As the increase in testosterone concentrations may stimulate pheromone production and libido, this may be part of the mechanism of social facilitation.

In accordance with observations by Sanford, Palmer & Howland (1982), LH levels were higher in inexperienced young rams than in experienced adult rams before ewes were introduced, and during the first 4 days (unpublished results). The decrease in circulating LH during the developmental period has been attributed to an increase of the feedback inhibition by androgens on the hypothalamo-pituitary axis (Foster et al., 1978; Wilson & Lapwood, 1979) and to the maturation of the negative feedback mechanism (Courot, De Reviere & Pelletier, 1975).

### *Maintenance of the response to the ram effect*

In Experiment 2 (**Paper III**), rams remained in contact with ewes until Day 55 and a second pregnancy diagnosis was performed on Day 74 in all ewes (unpublished results). In all groups, more ewes than those that were in estrus during the second estrus period (Days 17 to 25) were pregnant, which means that those ewes continued to cycle and came into estrus after Day 25. This differs from observations by Oldham & Cognié (1980) and Oldham, Pearce & Gray (1985), who reported that ewes that did not become pregnant during the first days after ram introduction failed to ovulate again. The continuous presence of rams is necessary to maintain ovarian activity (Murtagh et al., 1984b), and there is a direct relation between the proportion of non-conceiving ewes that return to anestrus and the expected interval remaining until the start of the breeding season (Oldham & Cognié, 1980). However, the stimulus obtained in our experiment was strong enough to maintain cyclicity of the ewes, although the study was performed far from the onset of the breeding season.

On the other hand, in the 2 experiments performed in laboratory conditions (**Papers I and II**) we did not observe any ewe coming into estrus after the initial ovarian responses (ovulation and/or luteal phase) after the introduction of the rams. They all returned again to anestrus without displaying estrous behavior during the observational period (23 days) and did not show another luteal phase after the initial one.

The differences observed in the maintenance of the reproductive response between laboratory experiments in **Paper I and II** and the field experiment (2) in **Paper III** suggest that our results were influenced by the locations where the experiments were conducted. Sheep show a stress response after transportation (Parrot, Hall & Lloyd et al, 1998) and

during the adaptation to a new environment (Gudev, Kolev & Madzharov, 1994). Although extensive attempts were made to minimize possible negative effects among rams in the laboratory experiments, it seems that these were not enough to eliminate a stressful response. In the field experiments, rams were transferred only between different pens in the same farm, which seems not to have caused stress.

### **The ram effect and estrus distribution in unprimed ewes**

In most of the field experiments we did not observe the classical bimodal estrous distribution in response to the ram effect. Actually, the unprimed ewes in the 3 experiments in **Paper III**, and in the **pilot study** (unpublished results) had an unusually delayed estrus concentration, coincident with the second estrous period despite that the experiments were performed in different farms, flocks, and categories (nulliparous and multiparous) of ewes.

In **Paper III**, the estrous distribution pattern showed that delayed ovulations followed by NLPs and SLPs followed by a second ovulation and an NLP were predominant. Few ewes responded with an early ovulation followed by an NLP. In that respect, results from **Paper IV** are contradictory. More unprimed ewes came into estrus between Days 17 and 20 than between Days 21 and 23. Despite the small number of animals, this different distribution could be explained by differences in body condition score (BCS) of the animals. Ewes used in **Paper IV** had very low BCS, mainly due to the rough weather conditions during the experimental period. The explanation could be that those ewes that may have responded with an initial SLP might have returned to anestrus without a second ovulation, which decreased the number of ewes in estrus during Days 21 to 23.

### **Priming of ewes with progestogens and the response to the ram effect**

In **Paper III**, we showed that short-term MAP priming may be used with results similar to those achieved by traditional long-term primings, and that as little as 20 mg of MAP in vaginal sponges is enough to ensure the same reproductive response as when 60 mg of MAP are used. This is an important advantage and gives flexibility when working in field conditions. It has been reported that the amount of MAP can be decreased in intravaginal sponges without affecting the reproductive results in cyclic ewes (Greyling et al., 1997; Simonetti et al., 2000), or in anestrus ewes stimulated with eCG after short-term priming (Ungerfeld & Rubianes, 2002) or with the ram effect after long-term priming (Rodríguez Iglesias, Ciccioli & Irazoqui, 1997).

Thus, the amount of MAP in commercial intravaginal sponges seems to greatly exceed minimum requirements. An important limitation in the knowledge of the pharmacological characteristics of MAP is the lack of available assays to measure the compound. We could only find one reference of MAP concentration measurements in relation to the use of intravaginal sponges (Greyling et al., 1994). In some experiments, as an approximation, the MAP amount remaining in the sponges after use was estimated (Greyling et al., 1994, 1997; Simonetti, Blanco & Gardón et al., 2000; Ungerfeld, 2002). Independently of the differences observed between experiments, the MAP amount which remains in the sponges after use is

high (Table 5). Usually, used sponges are discarded so to decrease the initial concentration in the sponges would be desirable to minimize environmental contamination with steroids.

Table 5. Maximum amount of medroxyprogesterone acetate that may be absorbed from intravaginal sponges according to initial content. Sponges were used for 12 to 14 days (<sup>1</sup> Simonetti, Blanco & Gardón, 2000; <sup>2</sup> Greyling et al., 1997; <sup>3</sup> Greyling et al., 1994; <sup>4</sup> Ungerfeld, 2002).

<b>Initial content (mg)</b>	<b>Final content (mg)</b>	<b>Maximum possible absorption (mg)</b>	<b>Maximum possible absorption per day (mg/day)</b>
59.1 <sup>1</sup>	34.3	24.8	1.8
44.1 <sup>1</sup>	24.3	19.8	1.4
39.6 <sup>1</sup>	18.0	21.6	1.5
56.3 <sup>2</sup>	47.8	8.5	0.6
36.9 <sup>2</sup>	22.8	14.1	1.0
49.1 <sup>3</sup>	46.6	2.5	0.2
35.9 <sup>3</sup>	26.1	9.8	0.8
25.0 <sup>3</sup>	16.4	8.6	0.7
60.4 <sup>4</sup>	56.2	4.5	0.3
35.5 <sup>4</sup>	32.1	3.5	0.3

We also observed that a single dose of MAP administered 3 to 5 days before the introduction of rams might substitute for a single dose of progesterone (Lindsay et al. 1984). Although there is a lack of information about the pharmacological characteristics (e.g., half-life) of MAP, our observations agree with previous reports of a long half-life of the compound compared with progesterone (Shelton, Robinson & Holst, 1967). Furthermore, when MAP was administered in the same way as progesterone – at the moment when rams were introduced – few ewes came into estrus before Day 28, but the number increased between Days 28 and 35 (unpublished observations). This suggests that the reproductive response was delayed and not blocked by MAP. In contrast to this observation, the percentage of ewes that came into estrus is not affected if progesterone is administered through an intravaginal device (CIDR) for 24 hours, beginning 1 day before or at the moment when rams are introduced (Pinczak & Ungerfeld, 2000).

### **Estrus period and fertility in progestogen-primed ewes**

In cyclic ewes, the fertility after spontaneous estrus is higher than in estrus synchronized with progestogens (Robinson et al., 1970). The lower fertility after progestogen priming may be due to a failure in sperm transport (Allison & Robinson, 1970; Pearce & Robinson, 1985) and/or the relation between progestogen levels and follicular dynamics (for review see Rubianes & Menchaca, 2003). The fertility observed in each estrous period in Experiment 1 (**Paper III**) is in agreement with those observations. Actually, ewes that came into estrus after a luteal phase with an endogenous corpus luteum (Days 17 to 28) had a higher conception rate than those ewes that came into estrus immediately after a MAP treatment. However, in **Paper IV** the results differ, with a lower conception rate in ewes that came into estrus after Day 17 than in those that came into estrus during the first 3 days. Again, the differences in results could be a consequence of the low BCS of ewes used in **Paper IV**. Those ewes that came into estrus and ovulated after Day 17 probably had one previous ovulation during the first days after the introduction of the rams. Since many ewes returned

to anestrus after the ovulation directly after ram introduction, one could expect that the fertility of the second ovulation would be low.

### **Response of cyclic ewes to the ram effect**

Our results in **Paper V** during the breeding season differ from those of AnnLai (1988), Ungerfeld & Rubianes (1999b), and Evans and colleagues. (2002). The diverging results could be attributed to the physiological status of ewes in relation to the period of the breeding season in which the experiment was performed. Ann Lai's (1988) and Ungerfeld & Rubianes' (1999b) experiments were performed at the onset of the breeding season. The experiment in **Paper V** was performed in the mid-breeding season, when ewes spontaneously display their maximum reproductive activity.

In **Paper III** (Experiment 2) we observed that the duration of luteal phases was related to their onset after ram introduction. Luteal phases that began late were shorter and luteolysis coincided with the beginning of the second estrous period. One could speculate that the mating activity may have triggered an increase in LH pulsatility and an increase in estradiol secretion with subsequent start of luteolysis. It would be interesting to test whether such a mechanism may exist in the late stage of the luteal phase of cyclic ewes. If so, it might be possible to synchronize part of a flock of cyclic ewes without the use of hormones by using the ram effect.

## Conclusions

- The concurrent use of ultrasonography and serum hormone concentrations resulted in the detection of a much wider range of reproductive responses to the ram effect than have previously been described.
- The growth status of the largest follicle alone does not determine the pattern of ovarian response of anestrous ewes to the ram effect.
- The endocrine environment in which a follicle grows, rather than the maximum size it attains, determines whether an ewe will respond to the ram effect or not.
- Progesterone priming allows a significant number of ewes to display estrus during the first days after the introduction of the rams and prevents the formation of short luteal phases.
- Short-term MAP priming may be used with results similar to those achieved by traditional long-term priming. The results are equivalent if MAP, FGA, or progesterone devices are used in short-term priming.
- The amount of MAP in intravaginal sponges can be reduced from 60 to 20 mg without affecting the reproductive response in short-term priming.
- A single administration of MAP applied 3 to 5 days before the introduction of the rams synchronizes estrous onset 17 to 18 days after rams are introduced.
- The patterns of FSH, LH, and estradiol-17 $\beta$  concentrations may be used as indicators of activity of the reproductive axis and to characterize anestrous depth.
- The ram effect is ineffective in inducing estrous synchronization in ewes during the mid-breeding season.
- The use of the ram effect, even without progestogen primings, seems to be an effective technique for out-of-season estrous induction in Corriedale ewes that can be incorporated in Uruguayan productive systems.

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No es aún lo peor errar en los accidentes del mar. Otros  
yerran por los malos documentos que se siguen  
Jorge Juan

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