

The Effects of Diethyl-Stilboestrol and Pregnant Mare Serum on the Oestrous Cycle of Merino Ewes.

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As is the case in other sheep breeding countries, it is a common experience in South Africa that the sexual activity of merino ewes may be suppressed for a period of several months each year. Where this period is unduly prolonged, it may, therefore, interfere with normal breeding operations.

In an extended series of investigations, Quinlan and Mare (1931), Roux (1936), Marais (1936), and more recently Quinlan, Steyn and de Vos (1941) found the anoestrous period in merino ewes under South African conditions to last approximately from September to December, i.e., during the first half of the summer season. In Europe, similar findings of anoestrus during the summer months have been recorded by several authors. According to Hammond, Hammond & Parkes (1942), the ovaries of such ewes are, however, not entirely quiescent during the anoestrous period seeing that enlarged Graafian follicles may at times be found in them and which in isolated cases result in actual ovulation, although not accompanied by any signs of clinical heat. This phenomenon of ovulation associated with so-called "silent heat" has been repeatedly observed by different workers, although its genesis as well as that of anoestrus itself still awaits final elucidation.

Concerning the aetiology of seasonal anoestrus in ewes it has been suggested (Parkes and Hammond, 1940) that this might be associated with a temporary insufficiency of gonadotrophic and follicular hormones. The resumption of normal pituitary and ovarian function in turn appears to be related with the nutritional state of the animals, evidence of which is afforded by the stimulating effect of "flushing" in contrast to the prolonged anoestrus displayed during periods of drought and poor feeding. According to van der Horst and Gillman (1942), anoestrus, the onset of which is a relatively acute process, inhibits the further formation of gonadotrophic hormones in the pituitary.

Apart from the possibility of these temporary fluctuations in the endocrine balance of the animal body, periodic changes in the reactivity of the reproductive organs, even to normal hormone stimulation, may in themselves be found to contribute towards the onset of the anoestrous state. In this connection the rôle exerted by nutritional and climatic factors, both on the level of hormone production as well as on the responses shown by the genitalia, constitutes an aspect of sex physiology requiring further investigations of a more fundamental nature.

With the object of inducing normal oestrous cycles during periods of anoestrus, hormone treatment in a variety of forms has, within recent years, been undertaken in different species of animals. As in other branches of endocrine therapy, the response elicited by such treatment has been found to vary with the nature and origin of the hormone used, with its dosage and methods of administration, and finally also with the physiological state of the body and the individuality of the animal.

In all this work two essentially different types of compounds have been employed throughout. These include (a) the *gonadotrophic hormones* as derived primarily from the anterior lobe of the pituitary, and in addition those encountered either in the serum of pregnant mares or in human pregnancy urine. According to present knowledge the two gonadotrophic hormones from the anterior lobe of the pituitary, viz., follicle stimulating and luteinizing respectively initiate and control ovarian rhythm and ovulation. In turn the two secondary hormones elaborated within the ovary, viz., oestradiol (formerly also known as folliculin) and progesterone, as derived from the corpus luteum, are known to be responsible for the various clinical phenomena associated with heat and the rest of the oestrous cycle, and thus also for the development and maintenance of the uterine and mammary tissues. (b) *Oestrogenic compounds* derived as naturally occurring hormones either from the ovaries, placenta, or from human pregnancy urine or alternatively as chemically synthesised compounds amongst which the highly potent oestrogen diethyl-stilboestrol has, through recent investigations, gained much prominence. Apart from their direct stimulating effects on the uterus, vagina and mammary glands, various oestrogenic compounds administered in adequate amount have been shown to exert a peculiar trigger-like action on the anterior lobe of the pituitary itself, a phenomenon which is as yet not fully understood [Steinach, Stäheli, Grüter (1934), Folley and Malpress (1941)]. According to Walpole (1941), the results of this so-called "oestrogen shock" depends upon the release of gonadotrophin from the anterior pituitary subsequent to a primary inhibition of this organ. Whatever the exact nature of the mechanism may be, evidence is accumulating to show that the judicious use of certain oestrogenic compounds are as effective in initiating ovulation and oestrus as are the gonadotrophic hormones themselves.

Seeing that the costs of production of highly potent stilboestrol is lower than that of the pituitary gonadotrophic hormones, the indications are that this synthetic compound will be extensively used in the treatment of certain types of functional derangement of the reproductive organs and especially in the initiation of the oestrous cycle in otherwise anoestrous animals. Accordingly a very considerable volume of literature has already been published concerning the action and uses of stilboestrol. Thus Montgomerie and Brownlee (1941) and Brownlee, Gould and Stuart (1942) record full oestrus in anoestrous heifers and cows following a single injection of 10-20 mg. of stilboestrol dipropionate. This is followed by a regular succession of normal oestrous cycles. Likewise Anderson and Bugg (1942) found that six anoestrous heifers were promptly brought into oestrus within 48 hours following the injection of 15 mg. stilboestrol dipropionate. Service at this stage, however, failed to induce pregnancy. Hammond, Hammond and Parkes (1942) record ovulation in the majority of anoestrous ewes following the injection of 0.5 to 1.0 mg. stilboestrol dipropionate. Both oral and vaginal administration of this compound failed, however, to produce these effects.

The results obtained with stilboestrol are essentially similar to those recorded from the use of oestradiol. Thus Steinach, Stäheli, and Grüter (1934) and Folley and Malpress (1941) were able to initiate regular oestrous cycles associated with fertile ovulation by the use of oestradiol (dipropionate or monobenzoate) on anoestrous heifers and cows. Likewise McKenzie and Terrill (1937) and Anderson (1938) record the onset of oestrus in the majority of anoestrous ewes treated with oestradiol benzoate.

Apart from the stimulating influence of stilboestrol on the genitalia, Folley and Young (1941), Lewis and Turner (1940) and Walker and Stanley (1941) noted a remarkable degree of mammary development in heifers and goats which in certain cases even resulted in copious lactation. According to Lipschütz and Vargas (1941) the exceptional activity shown by stilboestrol is probably associated with a greater resistance to inactivation in the animal body as compared with the natural oestrogens. From the findings of Sealy and Sondern (1941) the naturally occurring hormones, oestrone, oestradiol and oestriol were $\frac{1}{16}$, $\frac{1}{4}$, and $\frac{1}{40}$ respectively as active as stilboestrol when assayed by subcutaneous injection into test animals.

THE REACTION OF ANOESTROUS MERINO EWES TO STILBOESTROL.

In order to ascertain the effect of stilboestrol on merino ewes during the period of anoestrus, a series of experiments were undertaken at Onderstepoort in which varying amounts of stilboestrol were administered to a total of 43 full grown animals during the months of November and December. The complete absence of clinical oestrus amongst these animals, immediately prior to treatment as well as for several months preceding the experiments, was established by regular daily admission (morning and evening) of "teaser" rams amongst the ewes. Detailed records were kept of the oestrous response of individual animals. The brand of stilboestrol used was that supplied by Messrs. Boots, Nottingham. In all the tests, except one, the material was dissolved in oil and administered by deep intramuscular injection, the dosage ranging between 1 mg. and 5 mg. stilboestrol per sheep.

The following table indicates the results obtained from the different tests.

| No. of Animals Used. | Dose of Stilboestrol. | Reaction Displayed. |
|----------------------|---------------------------|---------------------------------------------------------------------------------------------------------|
| 12 | 5 mg. injected..... | 3 in full oestrus in 48 hours and served. Remaining 9—Negative. |
| 11 | 5 mg. dosed by mouth..... | All negative. |
| 13 | 2.5 mg. injected..... | 3 in full oestrus in 48 hours and served. 5 in weak, doubtful oestrus, not served. 5 remained negative. |
| 4 | 1.5 mg. injected..... | 1 in full oestrus in 24 hours and served. 1 in oestrus after 4 days. 2 negative. |
| 3 | 1 mg. injected..... | 3 in weak doubtful oestrus in 24-48 hours—None served. |

As will be noted from the above results 8 out of the 43 ewes treated with stilboestrol came into full oestrus, while a further 8 ewes displayed poor and doubtful signs of heat.

THE EFFECTS OF DIETHYL-STILBOESTROL ON EWES.

In order to ascertain the effects produced on the ovaries and uterus, two ewes which came into full oestrus 48 hours after the injection of 5 mg. stilboestrol were laparotomized on the 6th day following injection. In each case young corpora lutea were detected in the ovaries thus affording evidence of recent ovulation. The uterus in both ewes was pale and inactive. A further two ewes injected with 2.5 mg. intramuscularly, which had failed to display any signs of oestrus, were likewise laparotomized six days after injection. In one case there was a mature follicle (5 mm. diameter) present in one of the ovaries while the uterus appeared enlarged, turgid and hyperaemic. In the other ewe one of the ovaries showed a young corpus luteum in addition to an apparently mature follicle. Again the uterus appeared enlarged and hyperaemic. From the laparotomies thus performed it was evident that where oestrus was induced by stilboestrol, this was associated with ovulation and corpus luteum formation. Likewise there was definite evidence of follicle stimulation, ovulation, corpus luteum production and uterine reaction in two ewes despite the absence of any signs of clinical oestrus. These must be regarded as typical cases of ovulation with "silent heat" the aetiology of which as indicated before still awaits elucidation.

In addition to the above ewes treated during the anoestrous period, a further seven ewes were injected with stilboestrol (1 mg.) in April and May (i.e. during the height of the sexual season), in each case on the 8th day following normally occurring oestrus. Of these ewes only one came into full oestrus after 24 hours. This animal, together with two of the other ewes which failed to show oestrus, were laparotomized within 48 hours after injection. In all three animals there was evidence of recent ovulation as revealed by the presence of fresh corpora lutea together with definite enlargement and hyperaemia of the uterus.

As in the case of the anoestrous ewes, the results produced by stilboestrol on these seven normal ewes in dioestrus clearly indicate that ovulation is promptly induced by doses of stilboestrol as small as 1 mg. injected intramuscularly although in the majority of cases this is not accompanied by any signs of clinical oestrus. Similar experiences have been recorded by Parkes and Hammond (1940) with the use of horse pituitary extracts on anoestrous ewes and also by Van Aswegen and Quinlan (unpublished data) on the effects of prolan.

THE EFFECTS OF STILBOESTROL ON SPAYED EWES.

In order to test out the direct action of stilboestrol on the uterus and vagina, i.e., without the intermediation of any ovarian effects, five adult merino ewes were spayed in December, 1940. After a lapse of 12 months all the animals were injected with stilboestrol at varying intervals. The results of these tests are depicted in the following table:—

| Time of Injection after Spaying. | Stilboestrol Injected. | Results. |
|----------------------------------|------------------------|-------------------------------------------------------------------------------------------|
| 12 months..... | 5 mg..... | All 5 ewes in full oestrus in 24-48 hours allowing repeated service for further 2-3 days. |
| 16 months..... | 1 mg..... | All 5 ewes in full oestrus in 24-48 hours allowing repeated service for further 2-3 days. |
| 17 months..... | 0.5 gm..... | Two ewes in full oestrus in 48 hours. Three ewes remained negative. |

A subsequent injection of 0.3 mg. into the two ewes which gave a positive response with 0.5 mg. likewise induced full oestrus in both animals within 48 hours. The remaining three ewes which failed to respond to 0.5 mg. were later on injected with 0.75 mg. with the result that only one came into oestrus.

This experiment conducted on spayed ewes reveals the interesting finding that despite the loss of the ovaries 12 months previously, the remaining parts of the genital system showed an even greater degree of sensitivity to minute doses of stilboestrol than was the case in normal ewes. Thus doses of 1 mg. stilboestrol (and in some cases even less) promptly induced oestrus in all the spayed animals whereas in normal anoestrous ewes only a small percentage (8 out of 43) came into heat even with larger doses of stilboestrol, and despite the occurrence of ovulation which was regularly noted in laparotomized animals. In addition to the strong oestrogenic effects produced on the genitalia of spayed ewes, stilboestrol was also found to cause rapid development and turgidity of the mammary glands accompanied by actual milk secretion in two of the ewes. This confirms the findings of various other investigators previously referred to.

REACTION OF ANOESTROUS EWES TO INJECTIONS OF PREGNANT MARE SERUM.

Repeated investigations have shown that the blood serum of pregnant mares collected at a certain stage of pregnancy exerts powerful gonadotrophic effects when injected into non-pregnant females of different species. According to Day and Rowlands (1940), significant concentrations of gonadotrophic hormone appear in the blood of the mare only after 30-47 days of pregnancy. Subsequently it reaches its highest titre at 60-75 days, only to be followed by the disappearance of this hormone approximately 110 days after the onset of pregnancy. With the use of single doses of active serum, Cole and Miller (1939) were able to induce ovulation in anoestrous ewes which was unaccompanied, however, by any signs of oestrus. A further injection 16 days later was found to provoke both ovulation and oestrus. Bell, Casida, Bohstedt and Darlow (1941) similarly observed ovulation without oestrus in ewes treated with such serum. Cameron (1942) found the serum more effective on mares than on cows. From 11 anoestrous ewes each injected with 300-400 R.U. pregnant mare serum, 5 animals ovulated after 30-35 hours, although only one came into heat (McKenzie and Terrill, 1937). Hammond, Hammond and Parkes (1942) noted that all out of 11 anoestrous ewes killed 53 hours after the injection of 200-2,000 I. U. of pregnant mare serum had ovulated. These authors conclude that both anterior pituitary extract and pregnant mare serum evoke oestrus only in the presence of a regressing corpus luteum, while in the absence of this body, ovulation is not accompanied by heat. An active corpus luteum on the other hand suppresses both ovulation and heat.

With the object of studying the effects of pregnant mare serum on merino ewes under South African conditions, four mares were bled on the 90th day of pregnancy and equal quantities of the different sera subsequently mixed. This serum preserved with merthiolate (1:100,000) was used throughout in all the tests, which were conducted on a total of 110 adult merino ewes during the period October to December. All animals were in anoestrus during the whole of this period as shown by regular daily testing (morning and evening) with vasectomised rams. The following data was collected in the various experiments following subcutaneous injection of pregnant mare serum.

THE EFFECTS OF DIETHYL-STILBOESTROL ON EWES.

Experiment No. 1.—16 ewes injected 100 c.c. serum containing 100 mouse units per c.c., each on October 8th.

Result—Rams attracted by all ewes within 24-48 hours. Oestrus, however, incomplete as no ewe allowed service. Eight ewes slaughtered 72 hours after injection. In all cases ovaries found to contain enlarged cystic follicles up to 1 cm. in diameter although no ovulation recorded. Uterus in all animals only slightly turgid and vascularized although vulva definitely hyperaemic. Remaining 8 ewes came into normal oestrus following January.

Experiment No. 2.—10 Ewes injected 10 c.c. serum (1,000 m.u.) each on October 21st.

Result.—No definite signs of oestrus. Four ewes slaughtered 72 hours after injection. Ovaries in all cases enlarged and cystic but as in experiment No. 1, no ruptured follicles noted.

Experiment No. 3.—8 Ewes injected 0.5 c.c. serum (50 m.u.) in November.

Result—One ewe promptly in oestrus within 24 hours. Remaining 7 ewes showed no signs of oestrus, but vulva hyperaemic. From four of these slaughtered after 72 hours, three had definitely ovulated some hours previously, while the fourth animal showed mature follicle on point of rupture in one ovary. Three ewes not slaughtered came into normal oestrus in January.

Experiment No. 4.—35 Ewes injected 0.25 c.c. serum (25 m.u.) October 21st.

Result.—Although 4 ewes attracted the teaser rams, not one came into full oestrus. All ewes artificially inseminated 48 hours and again 72 hours after injection. None, however, showed pregnancy as all ewes came into oestrus during following January and February.

Experiment No. 5.—Late in anoestrous season (11th December) 41 ewes injected 0.25 c.c. serum (25 m.u.) while 30 untreated ewes remained as controls.

Result.—(a) 8 Treated ewes came into full clinical oestrus in 24-48 hours, 2 ewes in 5 days and 4 ewes in 8 days following serum injection, while all control animals failed to show oestrus. All treated ewes artificially inseminated 48 hours and again 72 hours following injection. Of these, only 4 ewes which showed oestrus within 24 hours became pregnant and lambled normally.

(b) 12 Treated ewes attracted rams but would not mate. External genitalia red and swollen. Together with control ewes these animals came into normal oestrus in January.

(c) 15 Treated ewes showed no sign of oestrus. Of these 9 were laparotomized within 3-5 days following injection. Fresh corpora lutea 24-48 hours old were found in the ovaries of 6 ewes showing that they had all ovulated very recently. In three cases the uterus appeared swollen and hyperaemic and the adnexia thickened and vascular while in the other three ewes only slight uterine changes were noticeable. In another one of the 9

ewes treated with serum a mature follicle was found in one of the ovaries, the uterus being enlarged and flaccid. The ovaries in the remaining two ewes were found to be small with the presence of atretic follicles in them.

DISCUSSION AND CONCLUSIONS.

There is ample evidence from the literature cited, that the various gonadotrophic hormones as contained in anterior pituitary extracts, pregnant mare serum, and to a lesser extent in human pregnancy urine, possess very definite follicle stimulating properties on the ovaries of various classes of animals, while in the anoestrous state. Similar results are achieved by the use of naturally occurring, as well as by synthetically prepared oestrogens, which through their effects exerted on the anterior lobe of the pituitary, likewise result in follicle stimulation. In the majority of cases this is associated with ovulation. Up to this point, our own data obtained from anoestrous merino ewes treated either with stilboestrol or with pregnant mare serum fully confirm the findings of other workers. Thus of all the treated ewes which were subsequently slaughtered or laparotomized, examination of the ovaries revealed either very recent ovulation in the majority of cases or the presence of mature follicles on the point of rupture.

With regard to the induction of full clinical oestrus and the willingness to mate, the results of the different investigations are, however, far more at variance. This is especially the case in ewes showing a well-defined seasonal anoestrus. In cows and heifers on the other hand where this is less evident, information thus far available tends to show that induced ovulation is more regularly accompanied by the onset of oestrus and the initiation of normal cycles than is the case in ewes. Thus, despite the high percentage of ovulation evoked amongst our experimental ewes, only 8 out of 43 treated with stilboestrol and 9 out of 110 which received pregnant mare serum came into heat sufficiently evident as to allow service. In contrast to this, the oestrogenic effects of stilboestrol in doses of 1 mg. intramuscularly were most decisive in the case of spayed merino ewes. Not only were all 5 animals used in the test repeatedly brought into heat after the injection of stilboestrol, but in every instance the onset of oestrus was both prompt, occurring usually within 24 hours, and fully sustained for a further period of 2-3 days, i.e., considerably longer than oestrus normally observed in ewes.

This finding affords strong evidence that the complete withdrawal of ovarian influences as achieved through spaying, provokes a definite increase in the sensitivity of the remaining genital tract to oestrogen subsequently administered. Conversely the oestrous response shown by a large percentage of anoestrous ewes was found to be completely absent when tested either with oestrogen (stilboestrol) or gonadotrophin (pregnant mare serum). No satisfactory explanation can as yet be offered for the wide fluctuations observed in the reaction of the uterus and vagina of ewes, which in the final instance forms the deciding factor in the onset of clinical oestrus. One possibility, however, which merits further investigation is the production of anti-hormone following upon repeated stimulation of the genital tract by oestrogen periodically released in the ovaries. According to this concept the phenomenon of seasonal anoestrus in ewes would then be explained not only on the basis of decreased gonadotrophic activity of the anterior pituitary as at present accepted, but equally as much on a temporary though cumulative resistance of the uterus to the full effects normally

exerted upon it by oestrogen. To what extent this fluctuation in uterine response is further influenced by such factors as age, breed, nutrition, climate and season is a matter awaiting more fundamental investigation. There is evidence to suggest, however, that the divergent results achieved by different investigators from the use of these hormones on anoestrous animals and especially on ewes, may be ascribed largely to differences in the physiological state of the individual animal at the time of treatment.

In this connection special reference should be made of the importance of the stage of anoestrus at which the investigations are conducted, seeing that different responses may be expected from animals during the early phase as contrasted to the end of the anoestrous period.

The observation repeatedly made, that ovulation as such could be readily induced by a variety of hormones during anoestrus, affords proof that the sensitivity of the ovaries to gonadotrophic influences is less effected than that of the uterus and vagina to oestrogen, whether this be naturally produced within the ovary or artificially injected. Moreover, the fact as pointed out by Hammond, Hammond and Parkes (1942) that the ovaries of anoestrous animals are not entirely quiescent during this period, indicates that gonadotrophic activity in the anterior pituitary is not completely suppressed, hence the explanation for the enlarged follicles which at times result in ovulation, although unassociated with heat (silent heat).

Concerning the influence of environmental factors on the anoestrous state, Zawadowsky and Margulis (1939), cited by the above authors, found that where a proportion of ewes came into heat spontaneously during a favourable year, their number could be considerably increased by a single injection of pregnant mare serum, while a second injection 16 days later evoked heat in 25 per cent. of the animals. The following year, however, when the ewes were in poor condition after a hard winter, and few came into heat spontaneously, neither one nor two injections of pregnant mare serum resulted in more than 1 per cent. of the animals taking the ram. These findings, in common with those of other workers, clearly indicate that the results to be expected from hormone therapy in the field of animal breeding, depend as much on the physiological conditions within the animal body at the time of treatment as on the nature and potency of the hormones administered. In this connection the degree of responsiveness shown by the anterior pituitary, the ovaries and particularly also by the female genital tube, forms the main deciding factors.

From the observations made on normal ewes as well as on spayed ewes following treatment with stilboestrol and pregnant mare serum, evidence is presented showing that seasonal anoestrus in merino ewes is closely associated with a decreased physiological response of the uterus and vagina to oestrogens.

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