**Breeding outcomes resulting from conception outside of the natural circannual breeding season – an Australian Merino sheep model.**

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**Objective**

To assess lamb survival and phenotypes when Merino sheep mate in synchrony or dissonance with their circannual reproductive breeding season.

**Methods**

Foetal and perinatal losses and phenotypes and their associations with conception time were examined. Lambs conceived at “in-season” or “out-of-season” matings were compared.

Melatonin was used to induce rams and ewes into breeding season activity during the light anoestrous period allowing comparisons in a randomized controlled trial.

**Results**

Relative Risk Reduction for foetal loss was 55% with rams and ewes induced into “breeding season” reproductive activity (75% for twins) with a predominant paternal influence apparent. Losses when ewes and rams had “out-of-season” activity were 29.7%.

Autumn conceptuses were twice as likely to be facial pigment free. 29% of autumn conceived progeny had high density and length fleeces compared to 8% of spring conceived sheep in one case study and 34% verses 22% in another.

Autumn conceptuses were 1.8 times more likely to be classed into the stud group compared to spring conceptuses and 80% less likely to be classed as culls.

**Conclusions**

“Breeding season” semen produced conceptuses that were less likely to die in-utero and perinatally. Autumn conceptuses were more likely to express better phenotypes for fleece density and length and freedom from facial pigmentation. There are clear advantages in avoiding dissonance between the circannual rhythm of breeding activity and the actual time of breeding.

The losses inflicted by “out of season” breeding is a potential source of huge loss as well as being an animal welfare issue.

***Keywords: Merino sheep, breeding season, circannual rhythm, melatonin, foetal losses, perinatal losses.***

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**Introduction**

Daily photoperiod, or more accurately, the rates of change of the ratios of light to dark exposure, has a marked influence on the breeding activity of sheep. Merino sheep exhibit maximum fertility and breeding activity during the period following the autumn equinox through to the winter solstice (“in season”) 1,2,3. Repeated studies in Australia have shown that Merino sheep produce up to 30 % more lambs if joined in the autumn rather than the spring and summer 4. The difference in seasonal fecundity has been thought to be attributable to the ewe, although scant data is available to confirm this.

However, at least 75 % of Merino joinings in Australia occur “out of season” in the spring and summer 5.  Anectodal evidence from pregnancy scanning operators indicate that at least 40 % of lambs in Merino flocks are lost in the period from pregnancy scanning at day 80 of gestation until lamb marking.

Daily photoperiod has a marked influence on the quality of semen produced by Merino rams. Semen collected from the spring equinox through to the summer solstice (‘out of season”) invariably shows signs of extensive apoptosis of the sperm 6. This cascade is triggered by ROS (reactive oxygen species) degradation. It is linked to DNA damage and fragmentation in the spermatozoon 7,8,9. Semen collected from the autumn equinox through to the winter solstice rarely show any apoptotic changes to the sperm 6. The high lamb losses may be occurring, at least in part, by using rams outside the breeding season. Genetic defects transmitted by degraded spermatozoa to the conceptuses or other epigenetic aberrations may be involved.

The possibility that lamb losses, prenatally and postnatally, may arise from “out of season” matings of rams is reported here. Also, the possibility that better phenotypes are likely to be produced when rams are joined during the peak fertility of autumn rather than in the spring and summer, was investigated. These hypotheses were investigated in several ways.

Firstly, the fate of progeny conceived from cryopreserved semen of a ram collected in the spring and autumn, and from natural matings of rams in the spring and autumn in two flocks, were monitored from early pregnancy through to lamb marking. In the cryopreserved semen study, return to oestrus was used as a de-facto pregnancy diagnosis and delayed return to oestrus was used as an indicator of likely embryonic or foetal loss.

Secondly, slow release melatonin (Regulin®; Ceva Animal Health), which induces reproductive activity that mimics “in season” reproductive performance in ewes 10,11 and rams 12 was used to compare foetal losses from treated and control groups of rams and ewes joined “out-of-season”.

Anecdotally, it appears that better phenotypes are likely to be produced when rams are joined during the peak fertility time of autumn rather than at any other time of the year. The association of “in season” and “out of season” conception time with resultant phenotype produced was also examined.

**Materials and Method*s***

Three types of studies were conducted**.** Firstly, the fate of progeny conceived from cryopreserved semen of a ram collected in the spring and autumn, were monitored from early pregnancy through to lamb marking in a case study. Secondly induced “in season” reproductive performance10,11,12 was used to allow comparison of foetal losses from treated and control groups of rams and ewes joined “out-of-season” in a randomized controlled trial. And thirdly, the fleece quality and facial pigmentation of progeny of the same rams joined in spring-summer versus autumn were recorded from two field case studies.

***Cryopreserved semen study***

Cryopreserved semen, collected from a stud Merino ram at a commercial artificial breeding centre in autumn (April, 1996) at the peak of the circannual breeding season, was used to inseminate 60 fertile, stud Merino ewes in May 1997. The semen had been extended and cryopreserved using egg yolk-Tris EDTA extender.

In the following May, the same 60 ewes were again inseminated with cryopreserved semen from the same stud Merino ram but on this occasion the semen had been collected and cryopreserved at the same facility using the same methods in October 1996. Collection time was in the depths of the circannual non-breeding season.

The thawed semen was assessed for peri-mortality apoptotic changes using the assay of Howe 6. This assay essentially detects membrane changes in spermatozoa that are associated with premature onset of apoptosis of the spermatozoa initiated by reactive oxygen species (ROS) generated aldehydes from the spermatozoa 8. It utilizes the B sub-unit of the heat-labile toxin of Escherichia coli (LT-B) to bind to the outer sperm membranes to assist detection of altered membrane structure and premature sperm death; affected cells being sorted by flow cytometry.

On both occasions of laparoscopic inseminations, the ewes were synchronized for oestrus using progestagen sponges (Chronogest 40 mg, Intervet ) and Pregnant Mare Serum Gonadotropin (PMSG) (Folligon 200 i.u., Intervet ) and laparoscopically inseminated 60 hours post-sponge withdrawal with no less than 80 x 106 spermatozoa per inseminate by intra-uterine injection.

The ewes were run with “teaser rams” fitted with sire sine harnesses for four oestrus cycles (68 days) post- insemination and monitored for returns to oestrus daily, with the cumulative numbers of ewes returning to oestrus tallied at the end of each 17day period.

***Melatonin study***

One thousand 16 months old Merino ewes, born and reared at “Nellup”, Wellington, New South Wales (latitude 330 south) were divided equally and at random into 4 groups. The ewes were of a single blood-line and were highly productive fecund sheep. Merino rams of similar breeding and reared under the same conditions as those for the ewes were divided randomly into 4 groups of 4 rams. The rams had been examined for breeding soundness and were proven productive fertile sires. Both rams and ewes were in body condition score 2.5 to 3 and were on a rising plane of nutrition.

The control group received no treatment. In group 2, the rams were treated with three slow release pellets each containing 18 mg melatonin (Regulin®, Ceva Animal Health). The dose of a total of 54 mg of slow-release melatonin was selected based on previous anecdotal evidence from results of off-label field use of the product in rams. In group 3, the ewes were treated with one 18mg pellet as recommended by the manufacturer. In group 4, the rams were treated with three pellets and the ewes treated with one pellet of melatonin. The pellets were placed subcutaneously at the base of the right ear.

After 42 days the ewes and rams were joined on 17 December 2013, two months before the commencement of the recognised breeding season for the Australian Merino in this geographical location 1, . Joining continued for 42 days and the groups were run in adjoining paddocks. The ewes were tested for pregnancy and assessment of foetal numbers using ultrasonography at 42 days after the completion of joining. All ewes were run together after the conclusion of joining through to weaning of the lambs at 4 months of age.

The flock was monitored closely through lambing to weaning and the lambs were accurately counted and identified for parentage.

Thirty three to 35 lambs born from each treatment group were randomly selected and a mid-side fleece sample collected from each animal. Fourteen to 15 of these lambs per treatment group were then randomly chosen for mid-side skin sampling.

A circular trephine, one centimetre in diameter was used to excise the skin samples after the skin surfaces had been clipped closely with fine scissors. The skin samples were stored in 10 % buffered formol saline.

Horizontal skin sections were cut using a modified form of the freezing technique of Nay 13, an electrical freezer being used on the microtome. For each lamb, the sections were used to measure diameters of 50 primary fibres, 100 secondary fibres, follicle density at 10 randomly selected sites on the skin section, and the secondary follicle to primary follicle ratios of 10 skin follicle groups.

The mid-side fleece samples of the unshorn lambs were used to measure the length of the outer fleece coat formed only by primary wool fibres, and the length of the undercoat fleece which consists of secondary wool fibres and primary wool fibres.

***Natural matings study***

Two trials were conducted.

In August 2013 at “Warwilla”, Wanganella, New South Wales, 106 Merino ewes, 12 months old and 267 Merino ewes,15 months old, were classed visually by one of the authors (JW) into a visual grade representing either high, moderate or low levels of fleece density and length using the method develop by Watts (unpublished). High density and length sheep have fleeces consisting of long and closely packed fibre bundles of high crimp amplitude and low crimp frequency. Low, density and length sheep have fleeces consisting of short, thick staples of low crimp amplitude and high frequency.

Facial and ear pigmentation was scored as either absent, moderate or excessive for each animal.

The ewes were the progeny of the same 18 rams and were carrying 7 months wool growth.

In November 2015, 1084 seven to 13 months old Merino ewes, at “Bundemar, Trangie, New South Wales, were classed by Robert Mudford into high, moderate or low grades for fleece density and length. The ewes were either conceived at the spring or autumn joining of their dams to the same 36 Merino rams.

**Statistical analysis**

Statistical analyses were carried out using IBM SPSS Statistics Version 22 (IBM Corporation). Odds ratios and Chi-square statistics were calculated for the incidences of the parameters observed. P < 0.05 was used as the minimum level of statistical significance.

**Results**

**C*ryopreserved semen study***

38.1% of the spermatozoa in the insemination doses of the spring collected semen were identified as having entered a premature cascade of apoptosis whereas the autumn collected semen, 6.3 % of the spermatozoa were affected. The LT-B labelled spermatozoa showed varying forms of tail and mid-piece deformities consistent with ROS induced damage.

The numbers of ewes returning to oestrus in each oestrus cycle period post- laparoscopic insemination are shown in **Table 1**.

After 3 oestrous cycles, the 60 ewes inseminated with spring collected semen were found to be 2.3 times more likely to return to oestrus than the same 60 ewes inseminated with autumn collected semen (OR = 2.27, 95% Cl = 1.09 to 4.73, p < 0.05). After 4 oestrus cycles, the ewes inseminated with spring collected semen were 3.5 times more likely to return to oestrus compared with those with pregnancies established with autumn collected semen (OR = 3.45, 95 % Cl = 1.63 to 7.30, p < 0.01).

The 60 ewes, when inseminated with spring collected semen, had a lambing rate of 35 % to produce 21 lambs. No lambs were retained as stud sires from this lambing. Most of the progeny were culled for excessive pigment on the face and feet and other physical abnormalities, such as hydrocephalus, agnathia, carpal deviations (bowed front legs).

The same 60 ewes, when inseminated with autumn collected semen from the same ram, had a lambing rate of 65 % to produce 73 lambs, of which 7 ram lambs were ultimately retained as sires. There were no birth deformities recorded and no body pigmentation reported.

***Melatonin study***

The survival rates of the foetuses detected at ultrasound examination at day 42 after the completion of the 42 day joining are given in **Table 2**.

The foetal progeny of melatonin treated rams were 35% less likely to die in-utero (RR= 0.65, 95% CI = 0.48 to 0.88), p <0.01) compared to the controls.

The foetal progeny of the melatonin treated ewes were 28% less likely to die in-utero compared to the controls (RR = 0.72, 95% CI = 0.54 to 0.96, p < 0.05).

When both rams and ewes were treated with melatonin, the foetal progeny were 55% less likely to die in-utero compared to the controls (RR = 0.45, 95% CI= 0.32 to 0.63, p < 0.0001).

Foetal twin progeny from conceptions where the rams alone had been treated with melatonin were 75% less likely to die in-utero compared to those from the untreated controls (RR = 0.25, 95% CI = 0.14 to 0.42, p < 0.0001). When ewes only were treated with melatonin twin foetuses were 44% less likely to die in-utero (RR = 0.56, 95% CI = 0.39 to 0.81, p < 0.0001). Treating both rams and ewes with melatonin resulted in twin foetuses being 75% less likely to die in-utero compared to the controls (RR = 0.25, 95% CI = 0.15 to 0.41, P < 0.0001).

When the non-surviving fetuses were considered as a separate population, twin foetuses were 53% less likely to die (RR = 0.47, 95% CI = 0.50 to 0.92, p < 0.05) when the ewes had been treated with melatonin compared to the untreated controls.

No lamb losses were recorded during lambing and weaning.

The fleece and fibre measurements of the unshorn lambs are shown in Table 3.

The mean outer fleece lengths for the 4 treatment groups were significantly different (ANOVA. F29= 1.589, p< 0.05). Outer fleece length was greatest in the progeny from the group where both the rams and ewes were treated with melatonin (mean outer fleece length 24.4 mm) followed by the progeny from the group where rams only were treated with melatonin (mean outer fleece length 21.7 mm). The diameters of the primary fibres that make up the outer fleece coats were significantly finer in those progeny from groups where the rams had been treated with melatonin (ANOVA, F 3=3.982, p = 0.012). There were no significant differences in the diameters of the secondary fibres (Ds), follicle density or secondary follicle to primary follicle ratio between the progeny of the 4 treatment groups.

***Natural matings study***

The numbers of progeny classed into high, moderate and low grades for fleece density and length in the two case studies are listed in **Table 4.**

In the Warwilla case study, 29 % of 106 autumn conceived sheep had high density and length fleeces, compared with 8 % of the 262 spring conceived sheep. This difference was statistically highly significant (p < 0.001). Also, significantly fewer (P < 0.001) of the autumn conceived sheep had low density and length fleeces (9 %) compared with the spring conceived sheep (36 %).

In the Bundemar case study, 34 % of 550 autumn conceived sheep had high density and length fleeces, compared with 22 % of the 262 spring conceived sheep. This difference was statistically highly significant (p < 0.001). Also, significantly fewer (P < 0.001) of the autumn conceived sheep had low density and length fleeces (7 %) compared with the spring conceived sheep (28 %).

Ewes conceived in autumn were 1.8 times more likely to be classed into the stud group (high density and length) compared to those conceived in spring (OR = 1.87, 95% CI = 1.43 – 2.45, p < 0.0001).

Ewes conceived in the autumn were 80% less likely to be classed into the culls (low density and length) compared to those conceived in spring (OR = 0.20, 95% CI = 0.14 – 0.30, p < 0.0001).

The numbers of spring and autumn conceived progeny from natural matings identified with facial pigment in the “Warwilla” case study are listed in Table 5.

The sheep conceived in autumn were twice as likely to be free of facial pigment compared to those conceived in spring (OR = 2.0, 95% CI = 1.2 – 3.1, p < 0.01).

Conversely, sheep conceived in the spring were twice as likely to have excessive facial pigmentation compared to those conceived in the autumn (OR = 2.04, 95% CI = 1.12 – 3.70, p < 0.05).

The sheep with excessive facial pigmentation were culled as unacceptable for breeding purposes.

**Discussion**

In a small study involving the cryopreserved semen of one ram and 60 ewes, it was shown that spring collected semen produced conceptuses that were 3.5 times more likely to die at the post-implantation embryonic and foetal stages than conceptuses produced from autumn collected semen. 64.9% of foetuses were lost within 68 days of conception and a further 9.6% by weaning following insemination with out-of-season collected semen. The spring collected semen had a high proportion (38.3 %) of sperm displaying apoptotic changes indicative of sperm dysfunction. The autumn collected semen was relatively unaffected (low apotopsis level of 6.1%).

In the cryopreserved semen study, the 13 surviving lambs from the spring conceptuses often displayed birth deformities and skin pigmentation problems. No ram progeny were kept. Thirty nine of the 60 stud ewes were rendered non-productive for the whole breeding season because the ewes had delayed returns to oestrus. In most artificial breeding programs, fertile, back-up rams are introduced post-insemination. With delayed returns to oestrus, resulting from foetal losses, opportunities to re-establish another pregnancy are also lost.

On the other hand, the 71 surviving lambs from the same 60 ewes, inseminated with autumn collected semen, had no birth deformities or skin pigmentation problems. Seven ram lambs were retained as stud sires.

The melatonin study points to the fact that endocrine and physiological states manifested naturally in sheep in the decreasing day length conditions of the autumn months, and mimicked by treatment with exogenous melatonin in our trials, are very influential on the survivability of the progeny. It should be emphasized that in the trials reported here, no attempt was made to optimize the melatonin dose, nor mode and regime of melatonin administration for the genotype of sheep involved. The results are therefore considered to be conservative rather than maximal.

In the melatonin study, foetal losses of 29.8 % in the untreated (control) group of sheep, which were by all definitions dissonant with their circadian and circannual rhythms for reproductive function when they were joined in early summer, is a huge production loss. We contend that this magnitude of foetal loss is representative of the losses being tolerated by the Australian Merino industry generally as a result of husbandry practices favouring out of season breeding.

We know that Luteinizing Hormone (LH) pulse frequency and amplitude are maximal under autumn conditions of decreasing day length 14. The resultant reproductive physiological states are associated with maximum numbers of ewes exhibiting oestrus and maximal ovulation rates. That foetal loss was reduced to 21.4% compared to 29.8 % in the untreated controls (a 28% Relative Risk Reduction for foetal loss) by inducing the ewes into reproductive activity akin to that of the natural breeding season is expected. We can argue that higher ovulation rates and more synchronized ovulations (commonly referred to as “the strength of ovulation” by sheep breeders) must result in better foetal and neonatal survival; but we have no direct proof of this. Nor have we any evidence that there were deleterious seasonal effects on oocyte developmental capacity with delayed cleavage and variation in gene expression that is suggested to occur in the bovine 15. Further investigation is required in this area.

What most would find unexpected was the finding that inducing the rams into a reproductive activity state consistent with that of the natural breeding season by administering melatonin, and joining them to ewes that were dissonant with their natural circannual breeding season, was associated with a Relative Risk Reduction for foetal loss of 35% leading to a foetal loss of 19.3% compared to 29.8% in the untreated controls. When both rams and ewes were induced into a reproductive activity state consistent with that of their natural breeding season, the Relative Risk Reduction for foetal loss was extended to 55% resulting in a foetal loss of 13.2% compared to 29.8% in the untreated controls. There is clearly an important paternal factor acting to increase the survivability of the foetus.

It was important for us to also focus on the foetal survival rates in twin pregnancies in the melatonin study because previous published studies 4 have suggested that additional lambs resulting from twin pregnancies when Merino sheep are joined at the peak of the natural breeding season are more often lost, thereby negating any advantage to discontinuing the practice of joining out of season. The Relative Risk Reduction rates for twin foetal deaths were 44%, 75% and 75% where ewes only, rams only or both rams and ewes respectively were induced into reproductive activity similar to that of the true natural breeding season. Again there is clearly a contributing paternal factor at play, as well as maternal factors involved. Considering the non-surviving foetuses as a separate population, twin foetuses were 53% less likely to die between pregnancy testing time and weaning when the ewes had been induced into breeding season-like reproductive activity with melatonin, demonstrating the importance of the maternal factors. One such factor is likely to be better pregnancy support by the corpora lutea 11.

No specific causal relationship between joining Australian Merino sheep out of season and the propensity for the foetus to survive has been elucidated by this study. We have however, clearly demonstrated an association, and speculating on a cause is hard to resist. We do know thatthe circannual rhythm of reproduction has a marked influence on the quality of semen produced by rams. Semen collected from the autumn equinox through to the winter solstice rarely shows any signs of the extensive apoptotic cascade triggered by ROS (reactive oxygen species) degradation 7 which is linked to DNA damage and fragmentation in the spermatozoon 8. ROS stress is a major contributor to defective sperm function 9. Such sperm dysfunction can lead to fertilization failure, failure to initiate and sustain normal embryonic development and the failure of establishment of immune tolerance of the conceptus by the female reproductive tract, in mammals 15, 16, 17. Semen collected in the spring or summer invariably has large percentages of spermatozoa showing signs of ROS induced degenerative changes 6.ROS affected spermatozoa induced into an apoptotic cascade and DNA damage, as well as the aforementioned dysfunctions, cause miscarriage and morbidity in the offspring 9, 18. It has been demonstrated, albeit in human semen, that none of the traditionally assessed parameters are correlated to the ability of the spermatozoon to fertilise the oocyte and the maintenance of pregnancies 19. The one single derived parameter that does correlate is the amount and quality of seminal plasma per spermatozoon in the ejaculate; and in seasonally breeding animals such as the sheep these vary enormously from autumn to spring and summer 6 and is involved in causing or suppressing the apoptotic cascades depending on the season.

We further argue that there can also be phenotypic deficiencies in the progeny, resulting in lost or diminished production traits when joining occurs out of season. Conversely joining ewes in autumn when the ram’s semen has very low levels of the ROS induced damage and the spermatozoa are fully potentiated, can avoid much of the losses. A number of phenotypic traits were examined in this study but the one reported here that is specifically associated with survival under harsh environmental conditions is that of birth coat characteristics 20,21,22,23,24. The outer birth coats (primary fibres) on the lambs from melatonin treated sires, which had the highest lamb survival rates, are the longest and also the finest.

Autumn conceptuses grew into sheep that expressed better phenotypes for fleece density and length and for freedom from facial pigmentation than spring conceptuses. We have shown that melatonin (Regulin (R) Ceva Animal Health), when administered to rams 42 days before joining and without optimizing either dose or treatment regime, reduced foetal losses from pregnancy diagnosis to weaning to by an absolute amount of 19.3% (13.2% when both ewes and rams were treated) compared to 29.8 % in the untreated “out-of-season“ controls. This may be one method for circumventing the losses and productivity wastage that is occurring in the Australian Merino enterprises; as well as addressing the animal welfare issues that are linked to the current breeding practices.

*Acknowledgements*

*We wish to acknowledge the invaluable help given by Robert Mudford from the Parkdale Poll Merino stud in providing sheep classing assistance as well as the East Loddon and Bundemar Merino studs. We are particularly indebted to David Pullen and family, Nellup Poll Merino stud, Wellington, New South Wales, for their continued help with the studies.*

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