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Factors influencing the incidence of anovulatory follicles in embryo transfer recipient mares

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1. Introduction

Ovulation failure whether it be part of a normal physiologic process, a pathologic occasion or result as a lack of response to an ovulation-inducing agent represents a significant cause of reproductive inefficiency in the mare. In a normal order of events a non-pregnant mare will return to estrous after being out of heat for approximately 2 weeks. From a group of follicles within a 'follicular wave' a single ovarian follicle will emerge and become a dominant follicle. Estrogens produced by this dominant follicle will fuel estrous behaviour, cause a cervical relaxation, stimulate edema within the uterus and increase secretion of luteinizing hormone (LH) from the pituitary gland, this LH surge will support the final maturation of the dominant follicle and induce ovulation. Sadly not every estrous cycle culminates with this ovulation (McCue, 2007; McCue, 2009).

It is difficult to predict if a dominant follicle in an estrual mare will be unsuccessful in ovulation as formation of an anovulatory follicle is usually preceded by development of normal endometrial edema and normal follicular growth patterns. Meaning the predicted formation of an anovulatory follicle and subsequent ovulation failure is impossible.

When organizing a breeding program for a mare, be it when to order semen or synchronization of recipient mares with a donor mare in an embryo transfer program, this inability to know when an anovulatory follicle will prevent ovulation can be detrimental to any breeding program resulting in both financial losses and loss of valuable time.

Anovulatory follicle development in the mare can be common during the transitional spring and autumn periods and despite being less common during the physiologic breeding season it is still a cause of ovulation failure and may be responsible for significant economic losses (Samper, J.C., *et al;* 2007).

Within embryo transfer (ET) incidence of an anovulatory follicle can be problematic for both donor mares and recipient mares. A successful ET program is reliant on both the successful breeding of the donor mare, with her consequent ovulation to produce an embryo and also successful transfer of that embryo into a synchronized recipient mare. The synchronisation of ovulation for at least two recipient mares per recorded ovulation of the donor mare is needed. Without synchronized recipients the production of that valuable embryo is pointless as there will be no suitable receptacle to transfer that precious embryo into.

Specific causes for the development of anovulatory follicles are unknown with hypotheses including insufficient estrogen production an inadequate LH surge and previous hormone treatments. Ovulation failure of this nature may also be associated with identifiable 'risk factors' such as advanced age, stress, certain medical conditions (such as Equine Cushing Disease, ECD) and drug therapy (McCue, 2009; McKinnon, *et al*, 2011). Older mares are renowned to have a higher incidence of anovulatory follicle formation (Samper *et al*, 2007). Vanderwall *et al* (1993) documented an age-related ovulation failure with mares, particularly over 20 years of age failing to ovulate despite showing oestrous behaviour.

The key to the future is to better understand the aetiology and causes of anovulatory follicles so that management can be based on prevention rather than post-formation treatment. The aims of this study were to look at meteorological data as a possible 'risk factor' in the incidence of anovulatory follicles. As of yet these variables have not been considered and therefore not studied, so this research aimed to initiate consideration of these meteorological variables as possible risk factors by a retrospective study into the climate at the time of anovulatory follicle detection.

The hypothesis of this research is that poorer weather during an active breeding season has an effect on the incidence of anovulatory follicles in the mare, with the belief that there will be an increase of incidence when the weather conditions are worst.

As little is known about the factors affecting the incidence of anovulatory follicles there is a potential relevance of this study to help the practitioner gain a better understanding of the possible causal factors. The study could also provide a useful insight for researchers willing to further investigate this phenomenon.

2. Literature Review

Despite the technologies of equine reproduction in today's world vastly increasing, we are still very reliant on the physiological process of ovulation and despite aids in its successful occurrence, ovulation failure continues to be a problem. Whether it be a natural service of your top thoroughbred racehorse, Artificial Insemination (AI) of an Olympic show jumping mare with frozen semen from another continent or synchronization of recipient mares for Embryo Transfer (ET) with a world class polo pony, in each of these cases ovulation is imperative for success. Unfortunately, not every dominant follicle results in an ovulation and even with modern day expertise ovulation failure if still a significant cause of reproductive inefficiency and subsequent economic losses.

Failure of a dominant follicle to ovulate and following persistence of an anovulatory structure has been reported in several domestic animal species and in women. This phenomenon has been described as naturally occurring in cows (Garverick, 1997), mares (Ginther *et al*., 2007) and women (Marik & Hulka, 1978). Research on methods of contraception and ovulatory process have also revealed experimentally induced unruptured follicles in rabbits, rats and women (Cuervo-Arango & Newcombe, 2010). Also structures of similar echotextual description to hemorrhagic anovulatory follicles in mares have also been documented from ultrasonographic examination in llamas (Adams *et* al, 1991) and women (Coulam *et al*, 1982). Similarly suggested is that haemorrhagic anovulatory follicles in mares may share some similarities with other anovulatory conditions in different species, such as; luteinized unruptured follicles (LUF) in women (Cuervo-Arango & Newcombe, 2009) and ovarian cystic disease (OCD) in cattle (McCue & Squires, 2002).

In the mare ovulation failure has been categorised by a haemorrhage into the dominant preovulatory follicle/s, which fails to rupture or collapse with subsequent organization of its contents and in the majority of cases luteinisation of the follicular wall (Cuervo-Arango &

Newcombe, 2009). This condition has been referred to in different ways, with an early reference to what seemed to be a similar structure in the 1940's (Burkhardt, 1948) when it was described as an occurrence of large persistent follicles during the months of October to November, later to be named 'autumn follicles' (Knudsen & Weiert, 1961).

An anovulatory follicle can be defined as: a dominant follicle with a failure to release its oocyte (therefore lack of its ovulation) not developing into a corpus haemorrhagicum or corpus luteum. Anovulatory follicles may also be called: ANOV's, Hemorrhagic Anovulatory Follicle (HAF), a Haemorrhagic Follicle (HF), a persistent anovulatory follicle, an autumn follicle and sometimes a Luteinized Unruptured Follicle (LUF).

Anovulatory follicles (ANOVs) of this nature have been given multiple names, being termed Haemorrhagic Anovulatory Follicles (HAFs) [Ginther *et al* 2007], autumn follicles (Knudsen & Weiert, 1961), hemorrhagic follicles (Ginther & Pearson, 1989) and persistent anovulatory follicles (McCue & Squires, 2002).

McCue & Squires (2002) reported that the majority of anovulatory follicles eventually become luteinized (85.7%), although some remain as follicular (nonluteal) structures (14.3%). Progesterone level testing can be used to determine the luteal status of these anovulatory follicles. This fate of the anovulatory follicles further categorizes them into luteinized (Figure 2) or non-luteinized unruptured follicles (Figure 1) depending on the degree of luteinisation of the granulosa cells and the progesterone secreting ability. The assessment of thickness of the granulosa layer of the anovulatory follicle and evaluation of its echodensity by ultrasonographic examination can be helpful to differentiate the presence of luteal tissue.

Figure 1: Ultrasound image of a persistent non-luteinized anovulatory follicle (McKinnon *et al*, 2011)

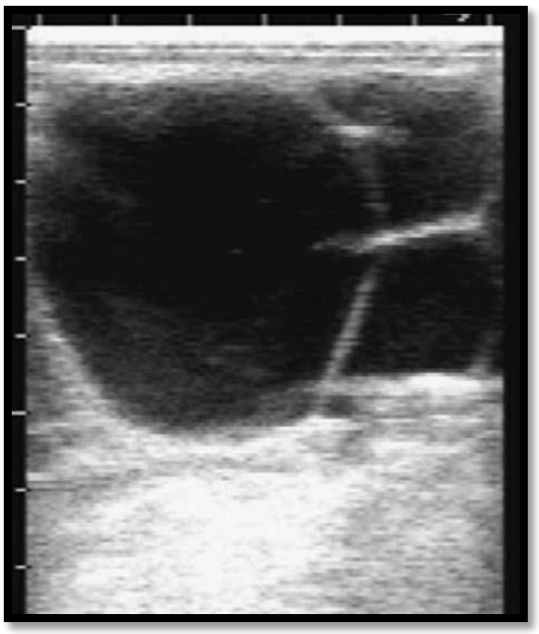
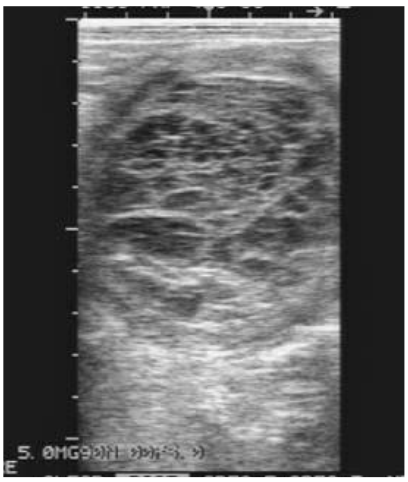


Figure 2: Ultrasound photograph of a luteinized anovulatory follicle (McKinnon *et al* 2011)



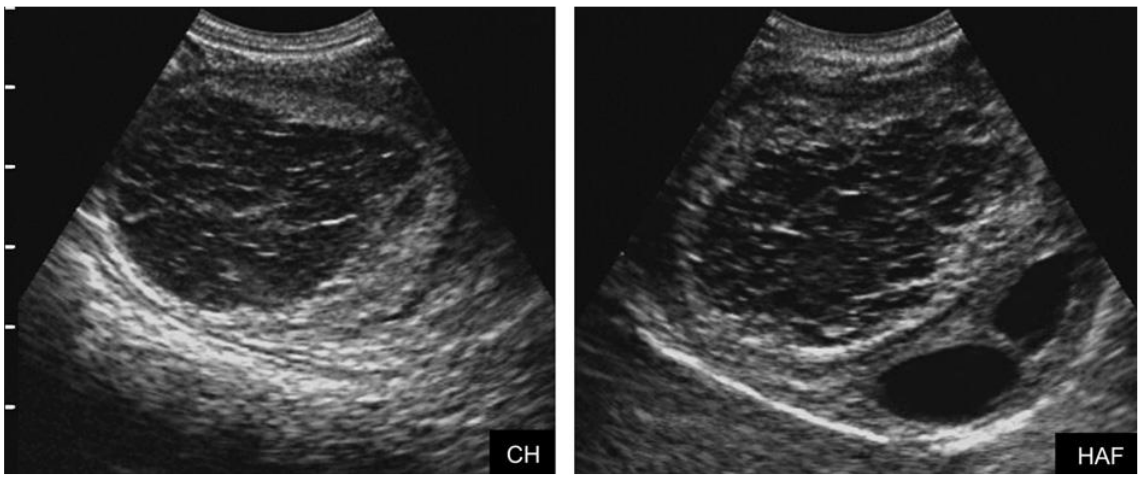
In human ultrasound and histological studies evidence was reported, that the development and ultrasonographic appearance of luteinized follicles happen in a similar way to that described in mares (Coulam *et al*, 1982). Resembling the condition seen in mares this disorder is termed Luteinized Unruptured Follicle (LUF) syndrome in women.

McCue (2009), reported the incidence of ovulation failure to range from 3.1 to 8.2% of all equine estrous cycles, with older mares having a higher occurrence of ovulation failure. About 13% of mares aged 16years or older experienced ovulation failure at least once during a breeding season and a high percentage (43.5%) of mares who developed anovulatory follicles had subsequent anovulatory follicle formation during estrous cycles of the same breeding season (Samper *et al*, 2007). Also seen was an increase in the interval of actual ovulations for mares that had developed anovulatory follicles during that season, this also can cause havoc with timings for breeding and synchronization programs.

Early detection of an anovulatory follicle is unlikely as it is difficult to determine in advance whether a dominant follicle is destined for ovulation failure. Formation of an anovulatory follicle is usually preceded by development of normal endometrial edema, initial follicular growth patterns are usually within normal limits and often the first indication of a problem is typically detection of the anovulatory follicle itself which is often too late to use that cycle.

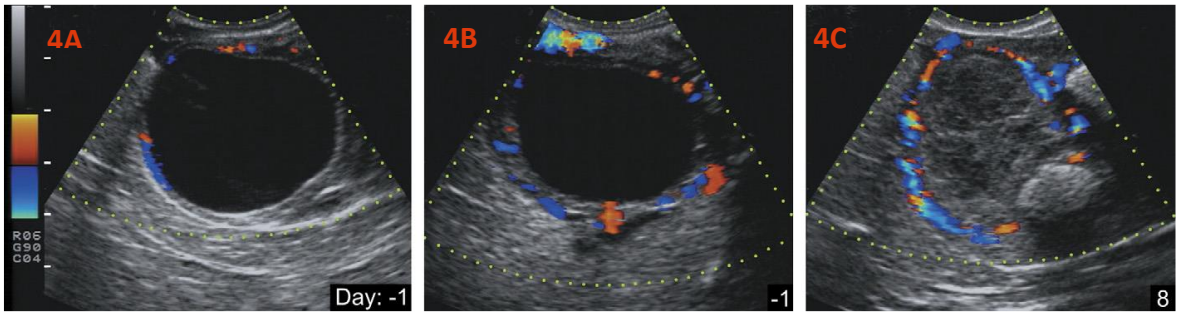
When diagnosing an ANOV it is important to perform multiple ultrasound examinations as measurements of individual structures on a given day may overlap between a Corpus Hemorrhagicum (CH) and a HAFs. Therefore size alone cannot be diagnostic and monitoring of the progression as well as the development of such structures is imperative to enable correct detection, as demonstrated by Ginther *et al* (2007) by ultrasonographic images to help to demonstrate that the morphology of a CH and HAF can be similar at certain stages (Figure 3).

Figure 3 Grey-scale sonograms taken 5 days after ovulation (CH) or anovulation (HAF) (Ginther *et al* 2007)



Ginther *et al* (2007) performed Doppler ultrasonographic examinations to look at the characteristics of the development of haemorrhagic anovulatory follicle (HAFs) in the mare. The study found only subtle differences in the follicular wall vascularity between ovulatory and HAFs during the 3days prior to ovulation/the beginning of haemorrhage (Figure 4).

Figures 4(A-C): Colour-Doppler sonograms showing blood-flow signals in the wall of the follicle on the day before ovulation (4A), the day before HAF (4B) and the HAF on day 8 (4C) (Ginther et al, 2007)



Not enough is known about the nature and factors affecting the incidence of haemorrhagic anovulatory follicles in the mare with some research into the time of the year, the age of the mares and exogenous hormone influences.

Hedberg *et* al (2007) highlighted some interesting points with their study into stress and the subsequent production of steroid hormones from the adrenal gland. Which were demonstrated as a potential cause of ovulation failure. They found with an increase in the production of cortisol, progesterone and androgens by the adrenal gland after administration of ACTH to intact or ovarectomized mares. Ferris & McCue (2010) also found that chronic treatment of cycling mares with dexamethasone resulted in an inhibition of LH secretion and resulted in ovulation failure. A similar effect was seen after administration of cortisol to ewes and gilts (Macfarlene *et al*, 2000; Scholten & Liptrap, 1978)

Cuevro-Arango & Newcombe (2010), along with others before them (Ginther & Pearson, 1989; Ginther *et al*, 2007; McCue, 2009) highlighted the need for better understanding of the risk factors leading to the development of Anovulatory Follicles in the mare. The objectives of their study were to define and characterize the ultrasonographic development

and incidence of HAFs and to investigate possible risk factors influencing its occurrence. They found that time of season and use of induction hormone treatments (cloprostenol) were found to influence the incidence of HAFs, with mares during the months of highest follicular activity (May-August) and after treatment with hormones to induce estrous and ovulation they were at a greater risk to develop HAFs.

In a recent study Burden *et al.* (2015) looked at the effect of cloprostenol administration on the interval to subsequent ovulation and anovulatory follicle formation in quarter horses. Despite the previous studies suggesting that exogenous PGF administration could be linked to the incidence of anovulatory follicles they found the overall incidence to be low (2.5%). This percentage is notably different from data published by Cuervo-Arango & Newcombe (2010) where an increase in incidence of HAFs formation was seen after cloprostenol administration in two mares (35.8% incidence rate) compared with untreated cycles (6.1%). Burden suggests that the differences in HAF formation between the two studies may be due to the number of mares in each study, breed difference of the mares, timing of the PGF treatment, dose of the PGF treatment administered, degree of reproductive management or geographic location. Perhaps climate differences should also be considered a possible reason for these differences and a contributing factor as to why HAFs were a less common observation in Burden *et al*'s study.

3. Materials and methods

3.1 Mares

Reproductive records were analysed for recipient mares kept at Beaufort Embryo Transfer (BET) Ltd in Gloucestershire, UK. Every recipient mare from 2006-2015 had their reproductive clinical record cards reviewed and were selected for this retrospective study accordingly (See 3.4). All the recipient mares were aged between 4-23years (with the majority being between 6-17years).

There was a variety of different breeds within the herd. All recipient mares resided at the centre living out at pasture 24/7 all year round, divided into groups of 10 to 25mares per paddock all on the same mixed pasture (mixture of different established grasses and legumes). Extra feed supplementation was not required during the active breeding season however haylage was fed additionally to the grass at the very beginning and end of the breeding season (March and October).

The majority of the mares had been included within the ET program at BET for multiple years and many had successfully been used as recipient mothers at some stage. For the purpose of this study no records were used from recipient mares with a foal at foot and once a mare became pregnant (successfully transferred and pregnant by embryo transfer) she was no longer scanned for ANOVs (just pregnancy scans) hence some mares "left the program" sooner than others.

All the recipient mares used in this study whether for anovulatory cycles or ovulation cycles were kept under the same environments constantly with no differences in these conditions.

3.2 Reproductive Records

The reproductive record cards of every resident recipient mare from 2006-2015 were reviewed. These records were always from between March to October with the majority

during June-August. Any mare that had an anovulatory follicle (fitting with the requirements (see 3.4) were included in this retrospective study.

Each clinical record contains the mare's name, age, observations for every reproductive rectal palpation and ultrasonographic examination usually carried out during the morning. These including, the date (time if multiple scans in one day), activity on left ovary, activity on right ovary, condition of the cervix (mainly determined by rectal palpation and scanning), comments on the uterus (any edema, liquid, pregnancy, cysts etc) and any comments, observations, procedures performed (cervical examinations, embryo transfers, Caslicks) and all drugs administered.

The daily reproductive checks were obtained by transrectal ultrasonographic examinations performed at least once daily during estrous and for at least two days after detection of ANOVs. The 3 ultrasound scanners remained the same over the 10years each equipped with a linear probe of 7.5MHz. All observations were recorded by a veterinarian whom was working under the supervision and guidance of BET's chief veterinarian, therefore all technicians were working to the same protocol and under the same training and specifications.

In many cases hormonal treatments were given to induce oestrus and ovulation (usually cloprostenol (Estrumate) and/or hCG (Chorulon)) their use was recorded in every case on the clinical card. Any other drugs given were also recorded on the clinical cards although they were used much less including; Oxytocin (Oxytocin-S) and sedation usually of xylazine (Chanazine) and/or acepromazine (ACP injection).

3.3 Ovulation

All recipient mare ovulations were recorded both in their clinical cards and in the synchrony book. As all recipients were being followed with the aim of synchronizing their ovulation dates with the donor mares this information was accessible and very reliable.

Detection of all ovulations were as per rectal palpation and ultrasonographic examination classified by the absence of a dominant follicle and the presence of a Corpus Hemorrhagicum (CH)/ corpus luteum (CL). With the day of ovulation recorded as the day in which it was first detected.

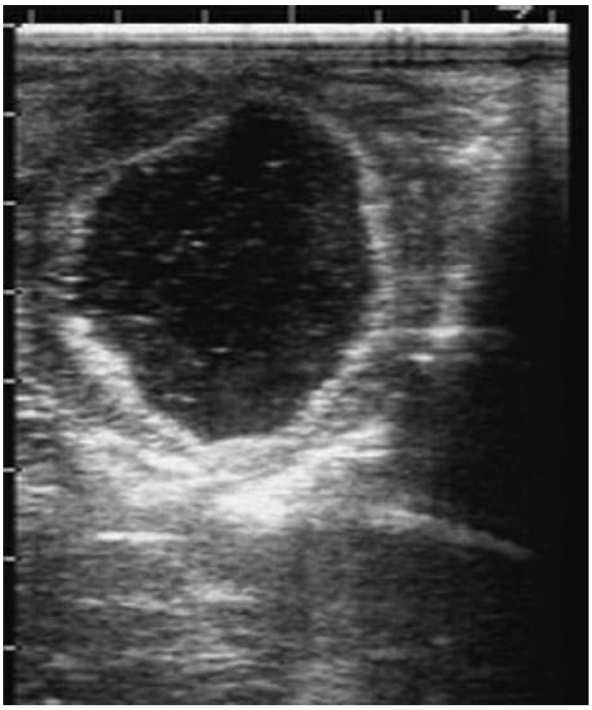
Every mare was scanned in the days leading upto and the days following all ovulations as was true for the mares who developed ANOVs as they too were being followed for monitoring of ovulation before the ovulation failure occurred.

3.4 Anovulatory Follicles

The data on anovulatory follicles were from the recipient mare records cards and were all obtained from previous rectal palpation and ultrasonographic examinations. All mares included in this study must have been scanned at least the day prior to the detection of the anovulatory follicle/s and scanned for at least two days post detection, for insurance that the follicle was definitely anovulatory. Anovulatory follicles recorded from only one ultrasonographic examination were excluded from this study due to previous studies highlighting the chance of an overlap in appearance of HAFs and CHs on a given day (Ginther *et al*, 2007) see Figure 3.

Detection of anovulatory follicles was described similarly to Samper *et al* (2007); scattered, free-floating echogenic spots within the follicular fluid detected during ultrasonographic examination of a dominant follicle with a failure to ovulate. The hemorrhagic follicles may be seen to contain echogenic fibrous bands or strands traversing the follicular lumen. The 'follicles' size may increase in diameter and eventually the contents acquire a static organized appearance. An example of some different anovulatory follicles can be seen in Figures 5, 6(A-C) & 7.

Figure 5: Ultrasound image of an anovulatory follicle showing multiple echogenic particles within the follicular lumen and a noted follicular wall thickening (McKinnon *et al,* 2011)



Figures 6 (A-C): A series of ultrasound images demonstrating the sequential events in the formation of an HAF A) Day 0; first day of HAF detection the follicle with excessive floating specks in antrum. B) Day 1; floating specks and the beginning of echoic strands. C) Day 2; a network of strands are seen. (Ginther *et al,* 2007)

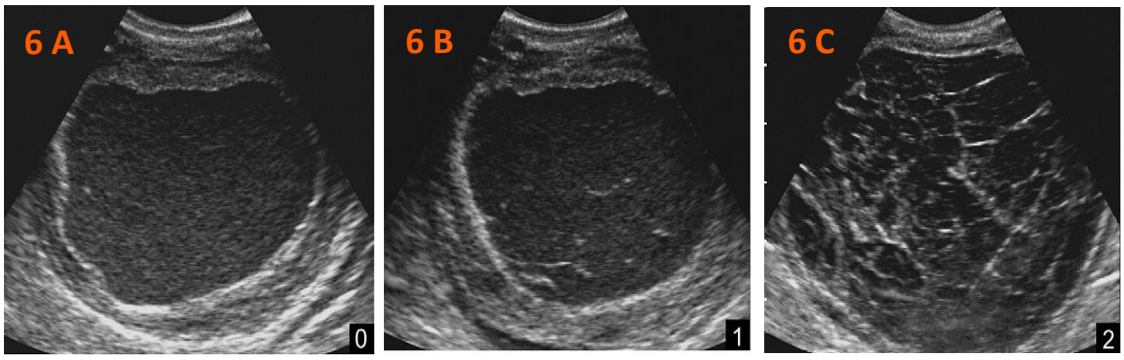
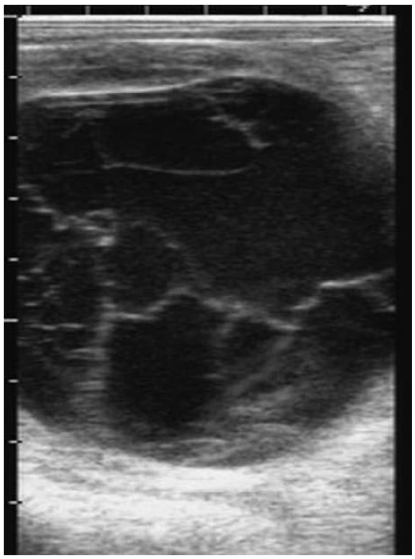


Figure 7: Ultrasonographic image of an anovulatory follicle with echogenic strands traversing the follicular lumen (McKinnon *et al* 2011).



3.5 Meteorological Variables

All meteorological data was collected from Gloucestershire and Nailsworth weather stations (Climate statistics, 2015; Historical statistics, 2006). There were 5 variables evaluated;

1) *Temperature low* (ºC),

2) *Temperature high* (ºC),

3) *Rainfall* (mm),

4) *Humidity low* (%),

5) *Humidity high* (%).

Each variable was recorded for the same day as the anovulatory follicle/s were first detected and evaluated.

3.6 Statistical analysis

Linear regression was used to analyse if there was any correlation between the log of the frequency of anovulatory follicles and the specific climate variable. Each individual variable was plotted against the natural logarithm of the incidence of anovulatory follicle/s on a scatter graph and insertion of a trend line aided in the visual assessment of the results. Both p-value and R^2 values were then calculated for each of the 5 variables to test the statistical hypothesis and the coefficient of determination respectively.

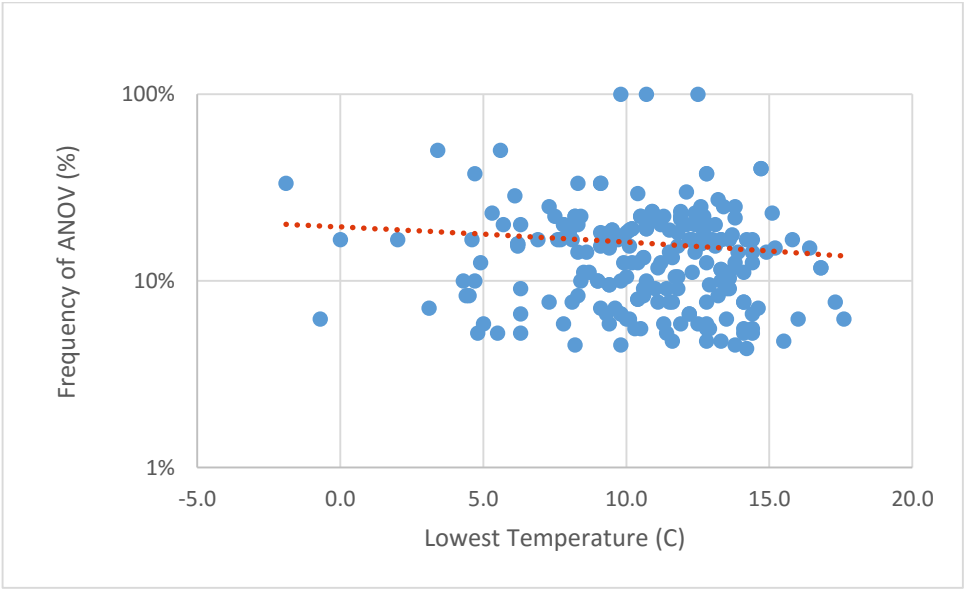
In order to calculate the frequency of natural logarithm for anovulatory follicles the number of 'normal' ovulations were used within ±2days of the date of ANOV follicle detection as were ±2days number of ANOVs.

4. Results

Of all the reproductive records studied retrospectively from 2006 to 2015 for recipient mares residing at BET Ltd, 95 mares were found to have had 214 anovulatory cycles. Mares ranged from having only 1 reported anovulatory cycle (45mares) up to 9 anovulatory cycles (9mares). The results showed no statistical significance however some visible trends were noticed with the linear regression suggestive that the *temperature high* variable may with further study be of interest.

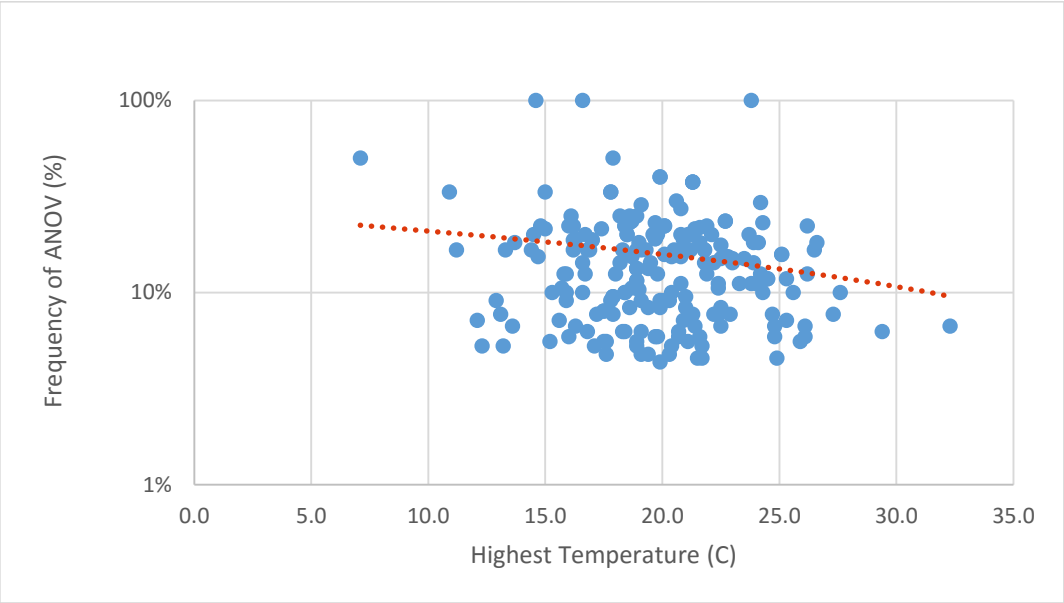
For the *temperature low* variable there was no significant correlation seen between this and the logarithmic frequency of anovulatory follicles (p=0.124). The distribution within the scatter graph does collect slightly around the trend line however it is also quite widely dispersed, with an adjusted R^2 value of 0.0066 (See Figure 8).

Fig. 8: Frequency of Anovulatory follicles in relation to lowest temperature (°C)



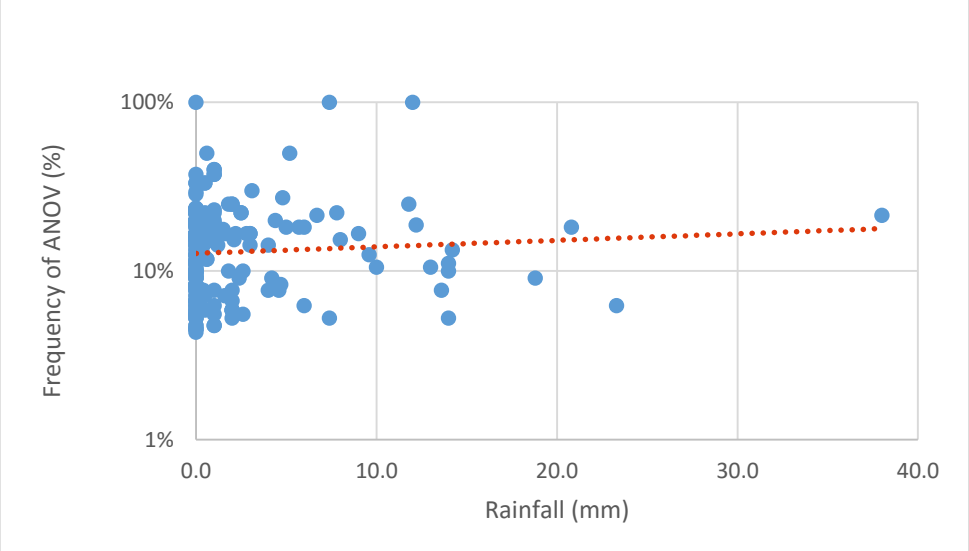
The closest and possibly most interesting findings were for the *temperature high* variable. Although the value was not significant on the log scale, it was very nearly significant (p=0.0673) and the distribution is visibly interesting on the scatter graph (Figure 9) where there looks to be a strong gathering around the trend line. There was an adjusted R^2 value of 0.0113.

Fig. 9: Frequency of anovulatory follicles in relation to highest temperature (°C)



Rainfall showed no visiable trends and no significant effect on the logarithm of anovulatory follicles (p=0.33) and an R^2 value of 0 (Figure 10).

Fig. 10: Frequency of anovulatory follicles in relation to rainfall (mm)



Neither of the humidity variables had any significant effect on the logarithm of anovulatory follicles either with both variants showing a wide distribution from their trend lines (Figures 11 & 12). *Humidity low* was not significant (p=0.382) and an adjusted R^2 value of 0. *Humidity high* was also not significant (p=0.172) and an adjusted R^2 value of 0.0042. Perhaps *humidity high* showing the most interest in this category with its p valve.

Fig. 11: Frequency of anovulatory follicles in relation to lowest humidity (%)

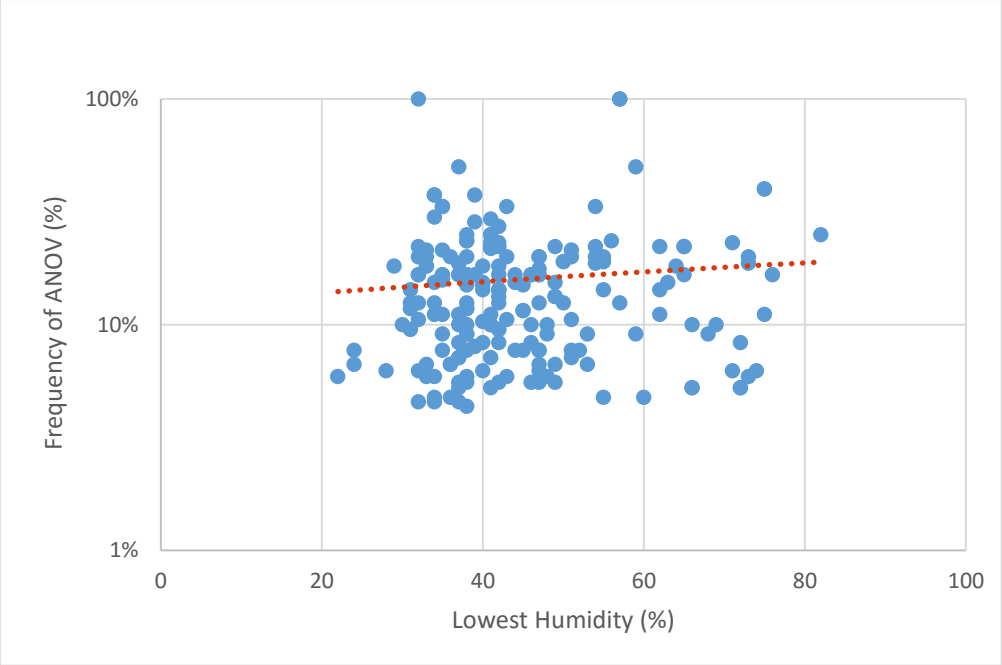
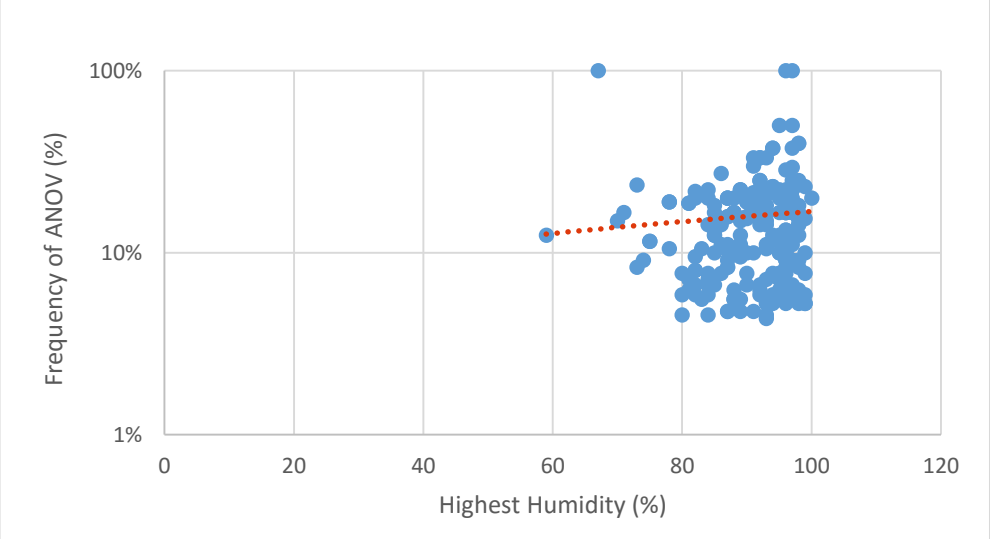


Fig. 12: Frequency of Anovulatory follicles in relation to highest humidity (%)



5. Discussion

This study intended to investigate a possible link between the incidence of anovulatory follicles and meteorological data. The reason for this type of ovulation failure is still not fully understood and no definitive cause of anovulatory follicles are known. Many have hypothesized towards possible causes and despite interesting and informative research stating probable influential factors contributing to the occurrence of anovulatory follicle/s it still remains unanswered and we continue to be unable to predict when a follicle is destined for this type of ovulation failure.

It has been suggested (Ginther *et al*, 2008; Cuervo-Arango & Newcombe, 2010) and it is of the authors opinion that the incidence of anovulatory follicles are a result of multiple factors, however by better understanding and identifying some of these possible 'risk' factors we can aid in limiting the contributing factors and hopefully reducing the incidence of ovulation failure due to anovulatory follicles. So that management can be based on prevention and not on post-formation treatment.

Although in the present study no significant correlation between the frequency of anovulatory follicles and the different meteorological variables was seen, when visually evaluating the data some trends were noticed. The *temperature high* variable does look to have some observable trend and possibly with a larger sample size perhaps a relationship would be significant. If we consider the nearly significant $p=0.0673$ and the visible trend (Figure 9) we can suggest that higher temperatures seem to decrease the chance of anovulatory follicles. The variance of this study is very high, so the effect of our data is much less than the effect of other unknown variables.

The basis for this study was due to observations by the author, during the years of 2002-2012 that during each breeding season when the weather was poorer (i.e. England was having a bad summer, with lots of rain and not reaching high temperatures) there was an apparent increase in the number of mares seen at Beaufort Embryo Transfer Ltd resulting with anovulatory follicles.

The main drawback with this study is the choice to only analyse climate variables from the day of detection of the anovulatory follicles. Also a suggestion for further research would be additional investigation of the meteorological data for the day's preceding the detection of the anovulatory follicle. The variables could remain the same but should definitely include the *temperature high, temperature low* and probably the *humidity high and low* also.

It was perhaps unrealistic with the data used not to imply an assumption that the anovulatory follicles will instantaneously develop on the day of detection, as this was not believed. Therefore by only analysing the weather variables from that given day, it was probably short sighted. However due to the lack of knowledge surrounding the true aetiology of anovulatory follicles the author decided that as a starting point for investigation the day of detection of these follicles was the only truly know definite value for preliminarily evaluating these anovulatory follicles.

To further this study weather data should be included from the days prior to detection of the anovulatory follicles (-6days, -4days, -2days would be suggested) as well as the day of detection (day 0), under the belief that if weather was having a influential effect on the development of an anovulatory follicle, that the cumulative effect of the days preceding its detection would be of interest.

Perhaps with further statistical analysis of the weather variables prior to detection of the anovulatory follicles a clearer more realistic account might be seen as to what accumulative effect the climate may be having on the incidence of anovulatory follicles.

With higher temperature believed to be decreasing the risk of anovulatory follicles it could be suggested that with further evaluation *temperature low* may also be seen to be having an effect. Looking also at the *humidity high* values achieved by this study they too could have some influence in a new study.

5.1 Clinical relevance and Conclusions

Temperature high appears to have a decreased chance of developing an anovulatory follicle. The visible trends seen in this study would be suggestive that if the weather is poorer (i.e: the temperatures are not reaching high values but remaining at the lower end of the high temperature scale. Perhaps with this in mind possibly by changing management aspects of the mares housing or rugging the mares at this stage might help. Or if the mare has a history of anovulatory follicles and the weather is worsening that you perhaps consider missing this cycle if the constraints of the breeding season allow.

It would also be interesting in future work to include the mares' age into the evaluations as this has previously been proven to be influential.

As some visible trends were seen, we could suggest that meteorological data may have some effect on the prevalence of anovulatory follicles. Higher temperatures appeared to decrease the chance of anovulatory follicles with this log-scale model being nearly significant. However, several other factors may still contribute to the incidence of these anovulatory follicles in mares.

6. Acknowledgements

The author would like to thank the kind and patient staff at BET Ltd for allowing me access to hundreds of mare records. My supervisor Professor Solti for his excellent guidance, support and for answering all of my questions promptly. Also to acknowledge professor Abonyi-Tóth for his patience and assistance with the statistical analysis. Finally my friends, family and especially Uilliam for their hours of tolerance and help.

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