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Reproductive tract disease and estrus detection inaccuracy in Irish seasonal calving pasture-based dairy cows

This thesis is submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D.)

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January 2022

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Thesis abstract

Many important factors impact reproductive outcome in Irish seasonally calving pasture-based dairy cows. Two of the factors that were investigated in this thesis were; 1) Reproductive tract disease (**RTD**) and 2) Estrus detection inaccuracy (**EDI**).

The study on RTD involved the retrospective analysis of 5,049 pre-breeding examination (**PBE**) records from 2,460 spring-calved cows on 8 farms over 5 years. PBEs analysed were conducted once between 25-86 days in milk (**DIM**) with two practical cow-side diagnostics which were;1) visual grading of vaginal discharge obtained with a Metricheck® device [purulent vaginal discharge (**PVD**) scoring on a scale of 0 to 3] and visual grading of ultrasound images of the uterus to assess evidence of endometritis [ultrasonographic endometritis (**UE**) scoring on a scale of 0 to 4]. The main objectives were; 1) to determine cow and calving related factors that were associated with increased risk of RTD, 2) to analyse the association between the test results of both diagnostics and subsequent reproductive outcome and 3) to the determine the diagnostic test parameters and select optimal thresholds for RTD at the time of the PBE. Multivariable logistic regression was used to identify factors that were associated with an increased risk of RTD when diagnosed by either PVD or UE scoring alone, or in combination. Sharded risk factors for RTD identified between both diagnostics were DIM at the at PBE, retained fetal membranes, twins, and cow genetics. Other risk factors that were specific for each diagnostic were dystocia, parity, BCS, breed, and ovarian findings. The association between PVD or UE score at the PBE and subsequent reproductive outcome was analyzed using Cox proportional hazards model. Cows with PVD score ≥2 (i.e. ≥mucopurulent discharge) and a UE score of score ≥1 (i.e. ≥ trace intrauterine fluid) were less likely to conceive than cows with healthy scores. To determine the test parameters and optimal thresholds for PVD and UE scoring for diagnosis of RTD at the time of PBE, a Bayesian latent class model was fitted. The optimal test thresholds selected were score ≥1 for both PVD (i.e. flecks of pus) and UE. At these thresholds, median sensitivity and specificity tend to be higher for UE than PVD but this was dependent on DIM at PBE.

Two observational studies were used to investigate EDI. In both studies, milk samples were collected on the same day as artificial insemination (**AI**) and analysed for milk progesterone (**MP4**) concentration using a radioimmunoassay. Cows that had a milk sample above a critical threshold of MP4 were determined to have been inseminated inaccurately, i.e., they were falsely diagnosed in estrus and AI'd. The main objectives of these studies were; 1) to estimate the cow-level true and apparent prevalence of EDI and 2) to determine potential cow-level risk factors for EDI. The first study involved the analysis of 576 milk samples from dairy cows in 125 herds whom received an AI on the same day as their routine milk recording. Using a Bayesian latent class model, the median estimate for cow-level true prevalence of EDI was 4.4% (1.7–9.0 %). In the second study, 1071 milk samples were obtained from 984 cows on 19 farms at the time of AI. Using multivariable logistic regression models that were stratified by insemination number (i.e., first versus repeat) cow-level risk factors for EDI were determined. Repeat

insemination had a higher apparent prevalence of EDI than first inseminations (14.1% vs 3.3%) and cows with no signs of mounting abrasions, had no observed standing estrus event or had an abnormal preceding interval since their last insemination were at increased risk of EDI at repeat insemination.

The results of this thesis will assist farmers and advisors in developing strategies that could help mitigate the negative impacts that both RTD and EDI have on reproductive outcome at cow-level but further research is required to validate these approaches.

Statement of original ownership

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work**.**

Emmet Kelly

January 2022

Preface

Globally and nationally dairy production faces many challenges notably producing food that is deemed sustainable and has minimal environmental or animal welfare impacts. The Irish dairy industry and producers aim to use the inherent advantage of our temperate location to meet these challenges by focusing on production of milk from pasture. To achieve this goal herd feed demand must be principally met by annual pasture supply and as such calving is seasonal. Consequently, maximizing reproductive performance in a time restricted breeding season becomes integral to this system. Understanding reproduction performance in Irish dairy cows in this system is an active area of research that encompasses a multitude of interlinked factors. Two important and under-investigated factors (i.e. reproductive tract disease and estrus detection inaccuracy) are studied in this thesis with the ultimate goal of broadening the knowledge base for farmers and advisors in these particular areas and hence contributing in some way to developing a more sustainable and productive industry.

As a background to this research, the normal and abnormal reproductive physiology of the dairy cow is reviewed in Chapter 1. Additionally, a review of how reproductive performance in dairy cows is measured and the complex interrelated factors influencing this performance is also provided in Chapter 1. Both Chapters 2 and 3 describe studies related to reproductive tract disease after three weeks postpartum, while Chapters 4 and 5 describe studies on estrus detection and insemination inaccuracy at the time of breeding.

In Chapter 2, some of the cow-level factors that are associated with the risk of developing reproductive tract disease are investigated. An analysis of the association between reproductive tract disease and reproductive performance in the study herds is outlined in Chapter 3. Additionally in Chapter 3, the determination of the diagnostic test parameters of two commonly used cow-side diagnostic tests (i.e. the visual assessment of vaginal discharge and the ultrasonographic assessment of uterine contents) for reproductive tract disease is performed. In Chapter 4, the cow-level true prevalence of estrus detection inaccuracy and thus inaccurate insemination (i.e., an insemination that takes place when a cow is not truly in estrus) in a population of in Irish milk recording dairy cows is determined while in chapter 5, some cow-level risk factors for estrus detection and insemination inaccuracy are investigated.

General conclusions arising from all these studies are discussed in Chapter 6. Based on the findings of the present thesis and literature review, practical recommendations have been made for farmers and their related advisors as well as recommendations and ideas for future research.

Chapter 1

Review of the Literature

1.0. Abbreviations used in this chapter

AI: Artificial insemination

BCS: Body condition score

BHB: Beta-hydroxybutyrate

CL: Corpus luteum

DIM: Days in milk

E2: Estradiol

EBI: Economic breeding Index

FSH: Follicle stimulating hormone

GnRH: Gonadotropin-releasing hormone

HF: Holstein friesian

IGF-I: Insulin-like growth factor 1

LH: Luteinising hormone

MED: Mating end date

MSD: Mating start date

NEB: Negative energy balance

NEFA: Non-esterified fatty acids

P4: Progesterone

PAGs: Pregnancy-associated glycoproteins

PGE2: : Prostaglandin E2

PGF2α: Prostaglandin F2 alpha

PMN: Polymorphonuclear Leukocyte

PVD: Purulent vaginal discharge

RFM: Retained fetal membranes

UE: Ultrasonographic endometritis

VWP: Voluntary waiting period

1.1 Dairy farming in Ireland

1.1.1 Irish milk production

In 2019, Ireland produced 7.9 billion liters of milk and was expected to exceed 8 billion liters in 2020 (BordBia, 2020). Despite this, Ireland is a relative minnow in terms of globally dairy production (0.94% total) (More, 2009). However, as we export 85% of annual production it is an important part of the national economy (3% of Gross Domestic Product) (More, 2009). In 2019, Irish dairy exports were the largest in the food and drinks category at €4.4 billion and these were largely in the form of processed dairy products such as butter, cheese, milk powders (skimmed milk powders, whey and casein) and importantly, infant milk formula (15% of global production) (BordBia, 2020).With such a focus on processed dairy products, most Irish co-operative pay farmers on a milk solids system (A + B - C basis) (Geary et al., 2010) with few having so-called 'liquid' contracts (approximately 10% of producers) (Veerkamp et al., 2002) . In this milk solids payment system, farmers are paid per kilogram of protein (A) and fat (B) produced and they are deducted a processing cost (C) in cents per liter (Geary et al., 2010). This payments system and our climatic conditions have been the major drivers for the type of dairy cow (i.e., lower yield but high milk solids output) and system (i.e., seasonal calving) that predominates in Ireland (Veerkamp et al., 2002).

1.1.2 Irish dairy farming systems and their relationship to reproduction.

The temperate climate of Western Europe facilities the growth of large quantities of grass over a long growing season (February-November) (Leaver, 1985) and thus pasture-based dairying is a natural fit for Ireland. Furthermore, these dairy systems have developed and expanded in Ireland in recent years due to their lower capital infrastructure costs, lower inputs per kilogram of milk produced, and the marketability of pasture fed dairy products (Roche al., 2017). The latter is an integral part of Irish export strategy with Bord Bia (Irish Food Bord) marketing Irish dairy cows as enjoying an 'average of 240 days per year out on pasture, with 95% of their diet consisting of grass (BordBia, 2020). To achieve this, herd feed demand must be mainly met by annual pasture supply and a relative small quantity of concentrates with the high dry matter intake of peak lactation corresponding with peak pasture growth rates (Dillon et al., 1995). When grass growth surpasses that of herd feed requirement in the summer it is conserved as grass silage and this forage is fed to dry cows over the winter period (November to January) (Dillon et al. 1995). The only way to accomplish the synchrony between grass growth and feed demand is to ensure that bulk of the herd calves in a condensed period or 'block' that occurs before the onset of the grass growing season (Figure 1.1) (Butler, 2014). In Ireland, this block calving period is from January to April, while in countries in the southern hemisphere with pasture-based dairy systems (e.g., New Zealand and Australia) it is from June to September (Holmes et al., 2002).

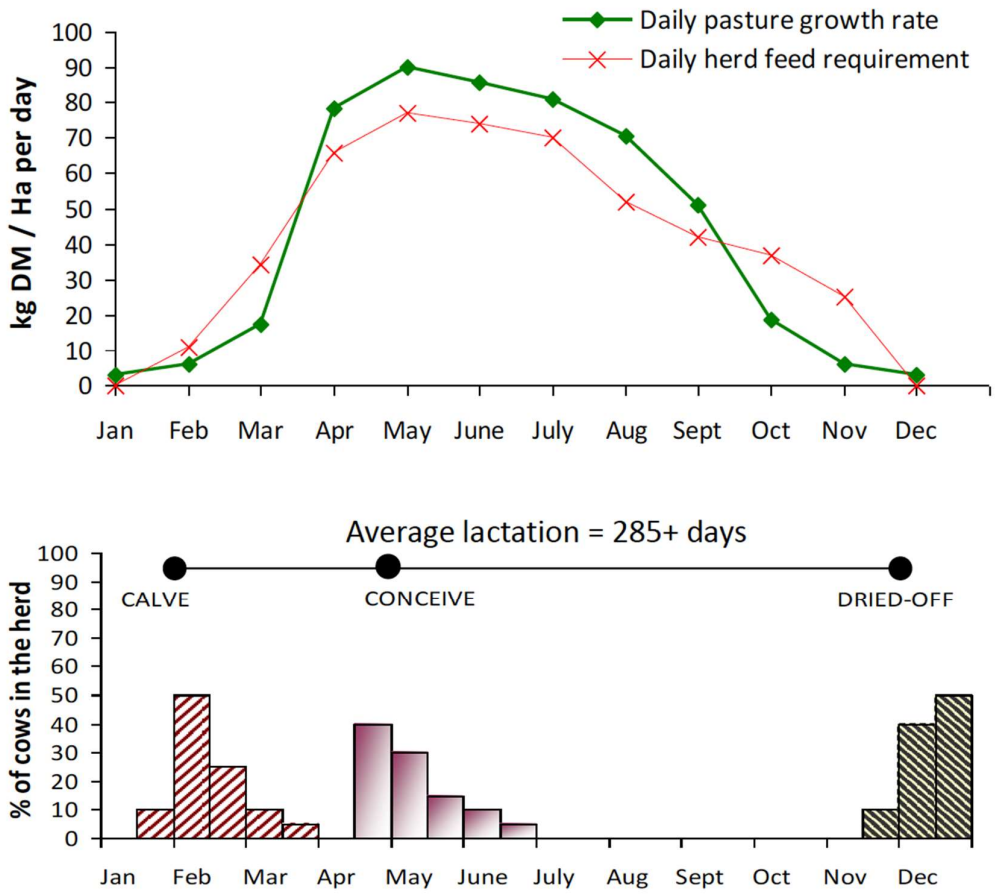

Figure 1.1. Typical calving and breeding pattern in a seasonal pasture-based dairy herd in Ireland [Butler (2014) adapted from Holmes et al. (2002)]

In order to operate such condensed period of calving, breeding is typically restricted to an intense 12-week period of work, commencing at a set calendar date (**MSD**: mating start date) in late April or early May, and ceasing at a set calendar date (**MED**: mating end date) in June or July in Ireland (Figure 1.1) (Butler, 2014). In the southern hemisphere seasonal calving systems, MSD and MED typically takes place in late September or October and in November or December, respectively (Holmes et al., 2002). The first 6 weeks of this intense breeding period is often managed by the farmer with estrus detection and artificial insemination (**AI**). AI used in this time is typically with dairy sires so replacement dairy stock will calve early in the following season (Berry et al., 2020). However, cows of inferior genetics may be bred initially to beef sires once the farmer is confident all replacement stock can be bred from his superior cows and nulliparous heifers (Berry et al., 2020). By the end of this period, the goal is to have all replacements pregnant and have the majority (>70%) of the herd pregnant (McDougall, 2006). The remaining 6 weeks are typically managed with bull breeding with the goal of achieving pregnancy

in the remainder of the herd (>90% at 12 weeks) (McDougall, 2006). Therefore, the importance that an individual cow's fertility plays when the breeding season is so time limited must not be understated. The importance of fertility is probably best highlighted by its prioritization (33% emphasis) as a genetic trait in the economic breeding index (**EBI**) set out by the Irish dairy industry, which is one of the highest genetic weightings in the world (Figure 1.2) (Cole and VanRaden, 2018).

Globally and nationally dairy production faces many challenges in the future such as the availability of skilled farm labor and farm automation alongside the animal welfare, environmental and sustainability concerns of consumers (Roche et al., 2017). Addressing these often-interlinked issues will grant what many authors refer to as, a "social license" to farmers to continue into the future (Roche et al. 2017). As reproduction is such an integral part of all dairy systems, but particularly in seasonal calving systems, further research into some of the factors that impact the reproductive performance of cows in these systems in Ireland is warranted and outputs are beneficial to improve animal welfare, longevity, profitability and sustainability.

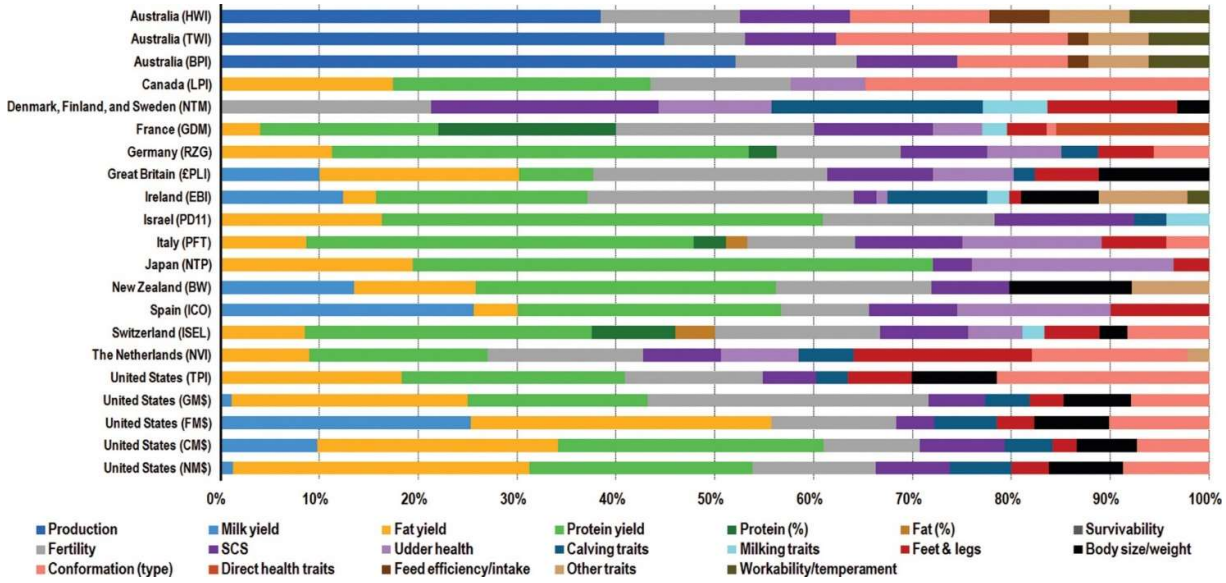

Figure 1.2. International Comparison of the relative weighting for breeding objectives in various National Dairy breeding Indices. Please note that the Irish Economic Breeding Index (EBI) has large trait emphasis on fertility (highlighted in grey bar) [Source; Symposium review: Possibilities in an age of genomics: The future of selection indices (Cole and VanRaden 2018)].

1.2 Reproductive physiology of the dairy cow

To understand the multitude of factors that impact the reproductive performance of dairy cows, it is initially important review the biological processes that mediate the following: the estrous cycle and the expression of estrus, the physiology of conception and gestation, the postpartum resumption of cyclicity and uterine restoration. Aberrations in this normal physiology resulting in reduced reproductive performance are also reviewed with reference made to the areas of focus of research in this thesis where appropriate.

1.2.1 Physiology of the estrous cycle and estrus in the dairy cow

Bos taurus cattle are the predominant dairy species outside of the tropics (Buchanan, 2016). These dairy cows are classified as non-seasonally polyestrous and thus display estrous behavior every three weeks (18-24 days) (Forde et al., 2011) once cyclicity has commenced post-puberty or postpartum. However, it is worth noting that regular cycles can occasionally cease for periods (Opsomer et al., 2000) or estrus signs can occur in non-ovulating cows (e.g., pregnant cows) (Dijkhuizen and van Eerdenburg, 1997). The estrous cycle itself can be broken down into two distinct parts: the luteal phase (also called met-estrus and diestrus) lasting approximately 14-18 days and the follicular phase (also called pro-estrus and estrus) which lasts approximately 4-6 days (Figure 1.3) (Forde et al., 2011).

Follicular growth in cattle takes 3-4 months and is divided into gonadotrophin independent (early) and dependent (later) stages of development (Webb et al., 2004). Throughout both follicular and luteal phases of the estrous cycle, waves of follicular growth (folliculogenesis) take place (Rajakoski, 1960, Savio et al. 1988) that are gonadotrophin dependent (Webb et al., 2004). Each wave of folliculogenesis consists of emergence of a group of follicles, selection of the dominant follicle from this group and then either ovulation or atresia of the selected dominant follicle (Sunderland et al., 1994; Ginther, 2000). Follicular emergence of a group of follicles (~ 5–20 follicles ≥5 mm) is commenced by a transient release of follicle stimulating hormone (**FSH**) from the anterior pituitary (Webb et al., 2004). Selection of the dominant follicle (~9 mm) from this cohort of emerged follicles occurs as a result of two simultaneous events in the soon to be dominant follicle (Beg and Ginther, 2006) and these are; the shift from FSH dependency to luteinizing hormone (**LH**) dependency [i.e., the Missouri Model; (Xu et al., 1995)] and the increased bioavailability of insulin-like growth factor I (**IGF-I**) in follicular fluid [i.e., the Cornell Model;(Fortune et al., 2004)]. The reason why these two developmentally critical events arise in one follicle over another is yet to be elicited but maybe due to advantages in the vascular bed, or the number and/or health of the granulosa or theca cells present (Lucy, 2007). Regardless of the reasons, these events allow for the continued growth, proliferation, and steroidogenesis in the dominant follicle. Once established the dominant follicle produces both estradiol (**E2**) and inhibin and both have negative feedback on FSH secretion at the hypothalamic-pituitary axis resulting in suppression of further

follicular emergence. However, the fate of the dominant follicle (i.e., ovulation or atresia) will depend on the prevailing LH conditions (Roche, 1996). In the early and mid-luteal phases, when progesterone (**P4**) is rising and elevated respectively, LH pulses are insufficient either in terms of amplitude or frequency to allow for final maturation and ovulation of the dominant follicle (Rahe et al., 1980). Consequently, the dominant follicle undergoes atresia, losing its steroidogenic capacity and FSH re-bounds to allow for new follicle wave emergence (Forde et al., 2011). Only in the follicular phase will basal P4 concentrations allow for adequate LH pulse frequency for final maturation and ovulation of the dominant follicle to occur (Rahe et al., 1980). Most dairy cows have two follicular waves per estrous cycle (Sartori et al., 2004) with follicular emergence at day 1 and 9 after ovulation. Dairy heifers tend to have three follicular waves per cycle (Sartori et al., 2004) with follicular emergence at day 1, 7 and 14 days after ovulation (Figure 1.3). However, there is considerable biological variation in individual cows and those with one or even four waves of follicular growth have been described (Savio et al., 1988).

The luteal phase of the estrous cycle follows ovulation and is the period of sexual quiescence. This period of sexual inactivity is the result of elevated P4 secreted by the corpus luteum (**CL**) which is a transient endocrine gland formed from an ovulated follicle (Forde et al., 2011). Although follicular waves continue throughout the luteal phase, the negative feedback that P4 exerts at the on gonadotrophin-releasing hormone (**GnRH**) and thus LH at the hypothalamic-pituitary axis means that estrous behavior is inhibited and LH pulse frequency is inadequate for ovulation and thus any dominant follicles undergo atresia (Lucy, 2007) (Figure 1.3). Post-ovulation, the CL has a grossly hemorrhagic appearance but by 48 hours it has attained a diameter of about 1.4 cm and by the 8th day post ovulation it has reached its' maximum size of somewhere between 2 to 3 cm in diameter, weighs up to 8.5 grams and obtains its' classic yellow appearance (Taylor and Rajamahendran, 1991, Mann , 2009).The volume of the CL is also directly related to the size of the pre-ovulatory follicle, with cows having larger ovulatory follicles and thus CLs than heifers (Sartori et al., 2004). The bovine CL consists of connective tissue cells (fibroblasts, smooth muscle cells, pericytes and endothelial cells), large luteal and small luteal cells, and the latter two cells originate from the granulosa and theca cells of the pre-ovulatory follicle respectively (Diaz et al., 2002). As well as differing in size (~38 μm versus ~17 μm), large luteal cells differ from their small luteal cell counterparts in terms of their steroidogenic mechanisms and capacity (Diaz et al., 2002). In small luteal cells, P4 production is stimulated by LH via the numerous LH receptors on these cells while in large luteal cells this P4 production is largely independent of LH and these cells contain more receptors for E2 and prostaglandin-F2α (**PGF2α**) (Wiltbank, 1994). In cows, close to 80% of the circulating P4 is produced by large luteal cells owing to higher basal P4 production and its independence from LH stimulation especially in the mid-luteal phase (Diaz et al., 2002). P4 production increases dramatically after ovulation increasing continuously until day 14 post-ovulation despite the fact that CL volume does not change dramatically after day 8 (Sartori et al., 2004).

This disconnect is believed to be the result of hyperplasia of large luteal cells and an improvement in their steroidogenic capacity (Parkinson et al., 1994).

Circulating P4 depends on P4 production by the CL, which in turn is influenced by its' development after the LH surge, the number of granulosa cells that luteinize into large luteal cells, and constitutive production of P4 by the large luteal cells (Wiltbank al., 2014). However, circulating P4 is also dependent on the rate of P4 metabolism which is predominantly controlled by liver blood flow (Wiltbank et al., 2006). Liver blood flow is increased in lactating dairy cows due to elevated feed intakes and thus circulating P4 tends to be reduced compared to non-lactating animals such as heifers and dry cows (Sartori et al., 2004).

Circulating P4 plays an important role in numerous parts of the overall fertility of the modern dairy cow. Low circulating P4 during the pre-ovulatory wave has been linked with lower fertility while low circulating P4 after ovulation have been associated with reductions in conceptus growth and elongation, decreased interferon-τ production, and poorer pregnancy outcome (Lonergan and Sánchez, 2020). Increased circulating P4 at around ovulation and AI due to incomplete luteolysis has also been shown to reduce the probability of conception (Wiltbank et al., 2014). Thus, optimization of circulating P4 concentration at various stages of the bovine estrous cycle continues to be a large area of ongoing dairy cow fertility research.

The luteal phase ends with luteolysis; the functional and structural degradation of the CL and in the absence of pregnancy, this occurs at approximately day 16 of the estrous cycle (Forde et al., 2011). In ruminants, the major luteolytic factor is PGF2α (Kindahl et al., 1976) and it is secreted by the uterus following the upregulation of oxytocin receptors in the endometrium (Robinson et al., 2001). During the luteal phase, P4 from the CL acts on its uterine receptors to block expression of E2 and oxytocin receptors in the endometrium (Kimmins and Maclaren, 2001). However, when there is no interferon-τ secreted by the trophoblastic cells of a conceptus, these P4 receptors begin to decline toward the end of the luteal phase (Spencer et al., 2004). This decline eliminates the block on uterine E2 receptors which subsequently leads to the upregulation of oxytocin receptors (Spencer et al., 2004). There is a positive feedback loop between PGF2α and oxytocin whereby oxytocin binds to its receptor in the endometrium which stimulates the release of high concentrations of PGF2α into the uterine vein (Knickerbocker et al., 1988). Initially, pulsatile release of oxytocin from the posterior pituitary at around day 14 of the estrous cycle acts on endometrial oxytocin receptors and induces the release of luteolytic pulses of PGF2α from the endometrium between 14-16 days of the estrous cycle (Arosh et al., 2016). This PGF2α release subsequently amplifies luteal oxytocin release which serves to further boost the luteolytic pulses of PGF2α (McCracken et al., 1999). This pulsatile PGF2α release is thought to be important and five PGF2α pulses of one hour duration over a period of two days is required to induce luteolysis in sheep (Arosh et al., 2016). Uterine PGF2α bypasses systemic circulation by travelling to the ipsilateral ovary via ovarian artery due to the anatomical relationship between the vein and artery and the difference in

concentration gradient, also referred to as 'counter-current exchange'(Ginther, 1974). Once at the ovary, PGF2α causes functional and structural degradation of the CL via a variety of different physiological pathways. Key molecules such as endothelin-1, cytokines (tumor necrosis factor-alpha, interferons) and nitric oxide release are known to; stimulate further local PGF2α and leukotrienes release, decrease P4 secretion by the CL and induce apoptosis, all contributing to the demise of the CL (Skarzynski and Okuda, 2010).

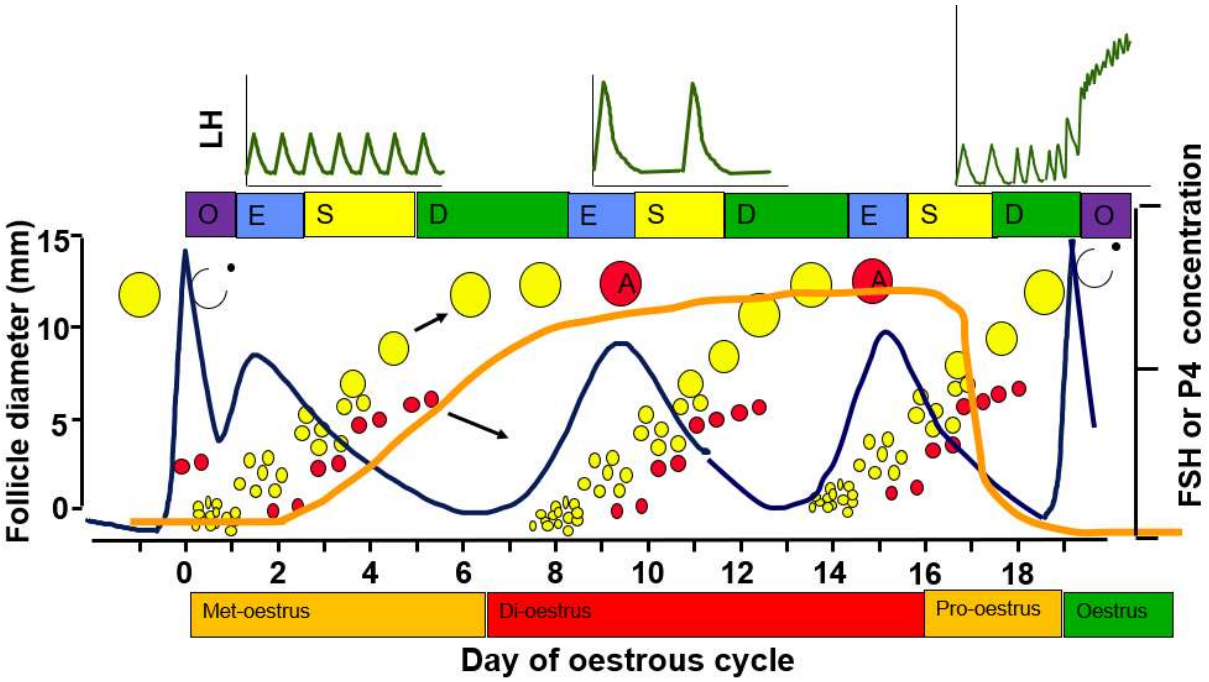

Figure 1.3. Hormonal and follicular events during the estrous cycle in the cow.

O = Ovulation of dominant follicle; E =Emergence of follicles; S = Selection of follicles; D= Dominance of follicles; A = Atresia of dominant follicle. FSH = Follicle stimulating hormone and is represented by the blue line: P4 = Progesterone and is represented by the orange line; LH = Luteinizing hormone and is represented by the green line. Please note this is an example of a cow with a 3-wave cycle with follicular emergence at day 1, 8 and 14 and atresia of the first two dominant follicles of the wave and ovulation of the dominant follicle of the third and how this is related to prevailing LH conditions (pulse frequency). (Source: Prof. Mark Crowe modified from Forde et al., 2011)

The follicular phase occurs after luteolysis, and it is during this stage, that continued growth, final maturation, and ovulation of the dominant follicle happens so a competent occyte can be released into the oviduct for fertilization (Forde et al., 2011). Following luteolysis, P4 concentrations are basal, and this removes the negative feedback that P4 has on GnRH and LH at the hypothalamic-pituitary axis (Forde et al., 2011). In this low P4 environment, elevated E2 from the pre-ovulatory dominant follicle

allows for positive feedback on the hypothalamic-pituitary axis to induce a GnRH surge and thus an LH and FSH surge which triggers ovulation in the dominant follicle (Sunderland et al., 1994) (Figure 1.3). LH pulses must occur every 40–70 minutes for at least 2–3 days before the dominant follicle will ovulate (Roche, 1996). This preovulatory LH surge triggers a range of physiological pathways such as local histamine, prostaglandin E2 (**PGE2**) and PGF2α release, a shift from E2 to P4 production by follicular cells, lysosome and collagenase production and increased blood flow and smooth muscle contractions (Murdoch et al., 1986; Niswender et al., 2000; Adams et al., 2008). This all contributes to weakening of the follicular wall and increased follicular fluid pressure which facilitates the eventual rupture of the follicle and release of the oocyte into the oviduct. The LH surge also plays a key role in oocyte maturation by allowing both the resumption of meiosis (from prophase I to metaphase II) and the cytoplasmic maturation necessary for a viable embryo (Assey et al., 1994). Ovulation of dominant follicles happens when follicles are between 12-20 mm (Ginther et al., 1996; Adams et al., 2008) and typically occurs 22-28 hours after the LH peak (Rajamahendran et al., 1993).

During the follicular phase, elevated E2 concentrations from the pre-ovulatory dominant follicle in a low P4 environment, allow for the cow to show behavioral signs of estrus (Forde et al., 2011) that farmers use to select dairy cows for AI. The duration of behavioral estrus in dairy cows is hugely variable with the mean duration somewhere between 6-10 hours with ovulation taking place approximately 28 hours after the onset or 10-12 hours after the end of estrus (Walker et al., 1996). In dairy systems, both the estrus duration and intensity are influenced by a range of animal factors (e.g., genetics, days postpartum, parity, milk production, lameness), environmental (e.g., housing, floor surface, external temperature/season, time of day, size of sexually active group) and management factors (e.g., farmer skill/diligence, time and frequency of observation, estrus detection aids) (Diskin and Sreenan, 2000; Roelofs et al., 2010). Although animal and environmental factors tend to have a true impact on estrus duration and intensity, management factors impact on the 'apparent' estrus duration and intensity, e.g., poor or infrequent observation may result in apparent short heats withing reduced mounting. Diskin and Sreenan (2000) noted that about 10% of the reasons for failure to detect estrus can be attributed to animal problems and 90% to these so-called "management" problems. Consequently, a deep understanding of these factors is important when advising on estrus signs and their detection in specific dairy systems.

1.2.2 External signs of estrus and methods of estrus detection

The external signs of estrus manifest as recognizable behavioral traits. The unequivocal primary sign of estrus in dairy cows is standing immobile while mounted by another cow or bull on the rear (Roelofs et al., 2010). The number of standing mounts per estrus event is as even more variable than estrus duration and anywhere between 3–140 (cows) and 3–225 (heifers) have been noted (Roelofs et al., 2010). Many secondary less reliable signs include mounting or attempting to mount other cows on the rear, mounting head side of another cow, increased activity, clear mucoid vaginal discharge, swelling and reddening of the vulva, increased body temperature, chin resting, head butting, sniffing of the vulva, flehmen,

bellowing, sweating and mild temporary reduction in feed intake or milk production (Roelofs et al., 2010). Both primary and secondary signs of estrus are used by farmers to select cows for AI. The signs are detected by frequent and direct observation of the aforementioned signs or through the use of simple, or more technologically complex, estrus detection aids which rely on specific behavioral signs.

Estrus detection aids that rely on mounting activity include tail paint, crayons, scratch cards and Kamars applied to the tail head or the use of radio telemetric pressure sensors or camera technology (Diskin and Sreenan, 2000; Palmer et al., 2010). The use of vasectomized bulls with chin balls also relies on this mounting activity (Diskin and Sreenan, 2000). Estrus detection aids that rely on increased activity include pedometers and accelerometers both utilizing an algorithm of baseline activity to determine when this activity increases above the baseline which occurs during an estrus event (Saint-Dizier and Chastant-Maillard, 2012). Other less common estrus detection methods include those that detect increases in body temperature (e.g., ear tag thermometers), decreases in rumination (e.g., boluses, accelerometers), impedance or conductivity measurements (e.g., vaginal mucous conductivity probes) and hormonal assays (e.g., serial inline milk P4 progesterone monitoring) (Diskin and Sreenan, 2000; Roelofs et al., 2010; Saint-Dizier and Chastant-Maillard, 2012).

Although, good stockmanship and regular frequent observations (approximately 4 times daily) of appropriate duration (at least 30 minutes) are still encouraged to detect estrus (Roelofs et al., 2010), traditional methods have significant time, labor and logistical challenges particularly in seasonal herds where cows are at pasture during the breeding season. Consequently, many automated sensors systems (i.e., automated detectors of standing estrus, pedometers, accelerometers and serial in-line milk P4 measurements) are now marketed to farmers for estrus detection and they have grown in popularity (Saint-Dizier and Chastant-Maillard, 2012).

To obtain optimal herd reproductive performance, whether automated or not, the ideal estrus detection method would include three elements and those would be; 1) the ability to detect a high proportion of cows that are in estrus; 2) the ability to correctly identify estrus (i.e., have no false activations) and 3) the ability to predict when those cows correctly detected in estrus were likely to ovulate (Hockey et al., 2010). Estrus detection performance of the two former goals can be measured in terms of two indices; estrus detection efficiency (or also called 'estrus or ovulation detection sensitivity') and estrus detection accuracy (or also called 'positive predictive value for estrus or ovulation diagnoses') (Morton and Wynn, 2010). Both measures can be determined based on serial and strategic milk P4 sampling throughout the estrous cycle and at the time of AI, respectively (Morton and Wynn, 2010). Estrus detection efficiency is defined as the proportion of estrus events that are detected while estrus detection accuracy is defined as the proportion of diagnoses of estrus that are true estrus events (i.e., ovulation is imminent and the cow not in the luteal phase) (Morton and Wynn, 2010).

However, the third element, i.e., the prediction of ovulation relative to onset of standing estrus can only be measured using serial ovarian ultrasonography (Hockey et al., 2010).

Many of the these methods described above can be logistically difficult to conduct on farms and are reserved to research studies assessing the utility of various automated estrus detection systems (Saint-Dizier and Chastant-Maillard, 2012) or to studies aiming to determine the performance of estrus detection in a population of cows (Sturman et al., 2000) or herds (Morton and Wynn, 2010). In a review by Saint-Dizier and Chastant-Maillard (2012), they found that the estrus detection efficiency of most of automated systems was greater than 80% but that the accuracy did vary considerably depending on the tool and algorithm developed. While the study of Morton and Wynne (2010) estimating the ovulation (or estrus) detection efficiency and accuracy found that both were generally high (median values of 94% and 97%, respectively) in seasonal herds in Australia. Farmer and advisors attempt to determine both estrus detection efficiency (i.e., via submission rate) and accuracy (i.e., via inter-insemination interval analysis) through analysis of reproductive data gathered on-farm and this is reviewed further in section 1.3.4.

In seasonal dairy systems, it is well recognized that estrus detection efficiency is essential to achieving herd reproductive performance targets (Morton, 2010). Although estrus detection accuracy is likely to have less of an impact on herd-level reproductive performance, research has shown that it can impact cow-level reproductive performance (Sturman et al., 2000; Lane et al., 2013). Although estrus detection efficiency has been quantified in some Irish herds (Jago and Berry, 2011), quantification of the level of estrus detection inaccuracy has not been studied in an Irish context and further research is warranted in this area.

1.2.3 Physiology of conception and gestation in the dairy cow

For a successful pregnancy to establish a competent oocyte must be fertilized by capacitated sperm, the embryo must develop from zygote to form a fetus with associated membranes and continue its' growth until parturition. The period from fertilization to parturition (gestation length) in the cow is typically a mean of 283 days. However, there is significant variation around this based on dam or sire breed and parity [e.g., Irish Holstein Friesian (**HF**) ~ 279 days vs Jersey ~283 days (ICBF, 2020a)].

Ovulation occurs 10 -12 hours after end of standing estrus and thereafter, it takes approximately 6 hours for the oocyte to traverse the oviduct to the site of fertilization (i.e., the isthmus and ampullary junction) which is approximately 30 hours after the onset of estrus (Roelofs et al., 2005). If mating or insemination occurs at estrus, sperm capable of fertilizing the ova reaches this site by about 8 hours (Hawk, 1987). The longer period that sperm have at this location allows for the selection of more competent sperm and best conception rates have been observed between 13 and 8 hours before ovulation (Nebel et al., 2000; Saacke et al., 2000) and is the main reason behind why the so called "AM-PM" rule that is advocated for the timing of AI. This rule describes the fact that insemination should take place in the evening if

the onset of estrus was observed in the morning and vice versa. At a practical level, establishing the exact time of the onset standing estrus is not always easily done so others now advocate that AI take place 6 hours after the initial observation of estrus (Nebel et al., 2000). The timing of AI based on the type of semen used for AI is worth noting, as the viability is reduced in particular types (i.e., sexed frozen <12 hours versus conventional frozen >24 hours) so AI closer to the time of expected ovulation yields a better probability of pregnancy (Bombardelli et al., 2016). Fertilization failure has been estimated to occur in 10-20% of AIs [Table 1.1; (Diskin and Morris, 2008; Wiltbank et al., 2016)] and has been attributed to many factors such as heat stress on gametes, incorrect AI technique or timing, uterine or oviductal infection or inflammation and incomplete luteolysis (Wiltbank et al., 2016).

After fertilization, the single cell embryo or zygote undergoes a series of mitotic divisions (also known as cleavage) to a 2-,4-,8 - and then a 16-cell embryo (Van Soom and de Kruif, 1998). By this cell stage or day 4-5 after fertilization, the embryo enters the uterus (Van Soom and de Kruif, 1998). While here it undergoes further cleavage to form a mass of cells where individual blastomeres become indistinguishable ('morula') (Van Soom and de Kruif, 1998). Once these cells form tight junctions, fluid accumulation is seen within the embryo ('blastocoele') and the embryo is called a blastocyst (Van Soom and de Kruif, 1998). It is at the blastocyst stage that two distinct cell types become visible, the inner cell mass, which will give rise to the embryo and the trophectoderm, which will form the placenta (Van Soom and de Kruif, 1998). During all these developmental stages, the embryo remains encased in the robust glycoprotein layer called the zona pellucida. However, by day 9-10 post fertilization the blastocyst hatches from zona pellucida and begins to grow (Lonergan and Sánchez, 2020). During this growth the shape of the embryo goes from spherical to ovoid (~6mm) and then the trophectoderm elongates to a filamentous structure commencing around days 12-14 post fertilization while the embryonic disc is minimally affected (Lonergan and Sánchez, 2020). This elongating conceptus reaches a length of about 60 mm by day 16 and is 200mm or more by day 19, almost doubling in length each day and extending into the non-pregnant horn (i.e., contralateral to the CL of pregnancy) (Brooks et al., 2014). It is during this exponential elongation phase, that the trophectoderm cells begins to produce interferon-τ, the maternal pregnancy recognition factor in cattle (Forde and Lonergan, 2017). Interferon-τ inhibits the process of luteolysis by inhibition of E2 and oxytocin receptor genes in the endometrial luminal epithelium and as previously mentioned, lack of oxytocin receptors in the endometrium will result in an absence of pulsatile PGF2α release (Forde and Lonergan, 2017). Outside of the zona pellucida, survival and development of the conceptus is dependent on maternally derived factors present in the uterine luminal fluid or 'histotroph' (Spencer and Hansen, 2015). Proteomic analysis of this histotroph has discovered many unique proteins with corresponding mRNA transcripts expressed from both the endometrium and the conceptus (Forde et al., 2015). This maternal-embryonic crosstalk is vital for embryonic survival and is predominantly regulated by maternal P4 as well interferon-τ and prostaglandins from the conceptus (Spencer and Hansen, 2015).

It is also during this exponential elongation that the key periods of gastrulation (germ layer formation) and onset of extraembryonic membrane differentiation begins (Brooks et al., 2014). During gastrulation, endodermal cells separate from the embryonic disc and line the inner blastocyst cavity while mesodermal cells from the primitive streak line between these endodermal cells and the trophoblastic cells. This happens at 18-23 days post-fertilization and forms what is called the trilaminar yolk sac and its' vascular part fuses with the maternal endometrium to form the choriovitelline placenta which it receives all nutrition via the yolk sac (McGeady et al., 2017). Once elongation and differentiation has been successful, the conceptus begins the process of central implantation and placentation around day 19 (Brooks et al., 2014). As the conceptus grows, a double layer of membranes that is formed by the trophectoderm and mesoderm becomes what is referred to as the chorion (McGeady et al., 2017). These layers also begin to envelope the conceptus, eventually meeting dorsally to encapsulate the fetus in what will be the protective fluid filled sac referred to as the amnionic sac (McGeady et al., 2017). Around the same time as amnion formation the allantois is formed as a diverticulum of the primitive hindgut and eventually expands as far as the chorion and its' vascular mesoderm fuses with the chorion to form the allantochorion (McGeady et al., 2017). The allantois functions to collect fetal waste while the vascularized chorioallantoic membrane in conjunction with the maternal endometrium will serve to deliver nutrients and a respiratory pathway for the developing embryo (McGeady al., 2017). The start of a switch from nutrition from uterine histotroph through a yolk-sac placenta to this chorioallantoic placenta that commences around day 28 and is complete by day 60 post-fertilization (Wiltbank et al., 2016).

The endometrium of cows consists of caruncles, round thickenings in the uterine mucosa (80-140 per cow), and intercarunclar areas. It is only in the carunclar areas that vascular attachments to the chorioallantoic membranes (cotyledons) occur with villous process invaginating to form the specialized physiological concave placentomes (McGeady et al. 2017). Although adhesive contact occurs at day 19, these vascular villi only begin to develop at around day 36 post-fertilization and are complete by day 60 (McGeady et al., 2017). Histologically, the placenta is classified as synepitheliochorial meaning that the endothelial, connective tissue and epithelial layers are intact and opposed both on the maternal and fetal side. However, in certain areas (crypt epithelium) of the placenta there is a combined maternal and fetal epithelium hence the term 'syn' implying union is used (McGeady et al., 2017). At parturition, the chorionic villi separate from the maternal crypts at the level of the microvilli and failure of to do so results in retained fetal membranes (see section 1.2.7).

Binucleate giant cells are an important part of the bovine placenta. They originate from trophoblastic cells and fuse with maternal crypt cells by day 40 and serve the purpose of transferring complex molecules between the fetus and dam (McGeady et al., 2017). These cells are also well known as they secrete the pregnancy associated glycoproteins or PAGs to maternal circulation and milk from day 28 and have been used widely for non-invasive pregnancy diagnosis in dairy cows (Zoli et al., 1992). The

placenta also serves as a source of supplemental P4 during gestation. Although the CL remains the main source of P4 through gestation a significant proportion is produced by the placenta between 5-7 months of pregnancy, but this source reduces again during the last months of pregnancy (Kindahl et al., 2004).

During the first trimester of fetal development, maximal placental growth, differentiation, and vascularization occurs, as well as fetal organogenesis, all of which are critical events for normal conceptus development (Reynolds and Redmer, 2001). However, in cows' fetal growth occurs exponentially especially in the last two months of gestation when over 60% of total growth occurs (Risco and Melendez, 2002). This growth results in a nutritional burden to the cow during the dry period and inadequate nutrition during this time has the potential to result in loss of body condition score (**BCS**) to maintain fetal development (Risco and Melendez, 2002).Conversely and as importantly, excessive nutrition during this time has the potential to result in BCS gain in the dam and placing the dam at high risk of experiencing dystocia and metabolic disorders in the postpartum (Risco and Melendez, 2002).

1.2.4 Aberrations in normal physiology of conception and gestation: Pregnancy loss in the dairy cow

The majority of pregnancy loss occurs during the early embryonic period, which is often defined as the period prior to maternal recognition of pregnancy (day 16) (Hansen, 2002; Santos et al. 2004; Diskin et al., 2006). This results in most cows repeating in the normal interval of days after estrus (18-24 days). There is a tendency for higher producing dairy cows to have greater levels of loss in this period of 28 days post fertilization when compared to more moderate yielding pasture based dairy cows (Table 1.1) with much of the loss in higher yielding cows occurring before day 7 (Diskin et al., 2011). After day 28 post fertilization, overall pregnancy loss is lower (i.e., 15-20% of all losses) with little evidence to indicate that this loss is due to genetics or level of production (Diskin et al., 2011). The majority of these later losses occur in the first 100 days with much less thereafter (Diskin et al., 2011).

Many genetic, physiological, nutritional and environmental causes of pregnancy loss have been implicated and are outlined in Table 1.1. Some of these causes have been well studied, others have limited evidence and other causes are yet to elucidated. Some of the better understood mechanisms are related to circulating P4 concentrations at various time points relative to ovulation, metabolic status of the cow postpartum, environmental heat stress and specific infectious pathogens such as bovine viral diarrhea virus, bovine herpesvirus (BHV-1), *Neospora caninum*, *Salmonella* Dublin, *Leptospira*, *Campylobacter fetus* among many others (Diskin et al., 2011; Wiltbank et al., 2016).

Low circulating P4 during the pre-ovulatory wave is linked to extended follicular growth of the dominant follicle, greater size at ovulation and diminished fertility (Savio et al. 1993; Cerri et al., 2009; Denicol et al., 2012). The oocyte of these larger dominant follicles may be less competent due to premature germinal vesicle breakdown because of prolonged LH stimulation (Revah and Butler, 1996) leading to many embryos that are considered degenerate at day 7 (Cerri et al., 2009). Adequately rising P4 concentrations post conception are known to induce temporal changes in the endometrial

transcriptome that promotes uterine receptivity and conceptus development (Forde et al., 2011). Consequently, low circulating P4 after conception have been associated with reductions in conceptus growth and elongation, decreased interferon-τ concentrations, and lower pregnancy probability (Lonergan and Sánchez, 2020).

Nutritional or metabolic status, especially in the early postpartum period, has long been suggested as a cause reduced fertility in modern dairy cows (Britt, 1991). During the early lactation when the energy required for milk production exceeds that of what can be consumed, a situation termed negative energy balance (**NEB**) ensues (Butler, 2014). The severity of this NEB will depend on the deficit with greater deficits resulting greater mobilization of body reserves (Butler, 2014). This will result in elevations in blood metabolites such as non-esterified fatty acids (**NEFAs**) and beta hydroxybutyrate (**BHB**) and reductions in IGF-I, insulin and glucose (Butler, 2014). Increase and reductions in these various metabolites have a widespread effect on the reproductive axis in the dairy cows but mostly manifesting in delays in resumption of cyclicity (see section 1.2.6) and reduced conception rates. It has been demonstrated that elevated NEFA levels are toxic for bovine granulosa cell growth in vitro (Vanholder et al., 2005) and that increased NEFA concentrations in follicular fluid affects oocyte quality (Leroy et al., 2005). As previously described, follicular growth takes 3-4 months in cattle and it is understood that these toxic exposures in early postpartum has lasting effects on oocyte quality once inseminated, the so called 'Britt hypothesis' (Britt, 1991). This lasting impact of NEB on oocyte quality and subsequent fertility may partially explain why more degenerate embryos have been noted in dairy cows with the greatest postpartum weight loss (Carvalho et al., 2014) and the negative association of BCS loss, BCS nadir and elevated NEFAs on the probability of pregnancy (Buckley et al., 2003; Roche et al. 2007; Ribeiro et al., 2013).

Although less of a issues in temperate climates, such as in Ireland, heat stress among many of its other detrimental effects on reproduction (e.g., reduced estrous expression and/or dry matter intake) can have lasting effects on oocyte quality and subsequent fertility (Rensis and Scaramuzzi, 2003). Heat stress impacts on oocyte function at two key periods of development; firstly, during genome activation and silencing in early follicular development and secondly, during nuclear and cytoplasmic maturation following ovulation (Hansen, 2009). This manifests as largely decreased capability of cleaved embryos to develop rather than the fertilized oocyte to cleave, ultimately leading to pregnancy loss (Hansen, 2009).

Table 1.1. Summary of pregnancy loss during in lactating dairy cattle.

Adapted from Diskin et al. (2011) and Wiltbank et al. (2016).

Days post-fertilisation	Fertilisation Failure	0-7 days	8-28 days	28-60 days	60-90 days	90 + days
Loss at time point (Wiltbank et al. 2011)	10-20%	10-50%	10-20%	5-20%	≤2%	NS
Loss at time point (Diskin et al. 2011)	10%	28-43%		7-10%		
Potential Reasons for Loss	• Heat stress on gametes • Incorrect AI technique/timing • Uterine/oviductal infection/inflammation • Incomplete luteolysis	• Oocyte problems • P4 before AI • Postpartum metabolic issues	• Ill-timed elongation • Low P4 after AI • Histotroph deficiencies	• Inadequate placenta formation • Inadequate vascular development • Underdevelopment • Infectious agents	• Unilateral twins • Infectious agents	• Infectious agents

1.2.5 Physiology of the resumption of cyclicity in the dairy cow

Follicular waves not only occur regularly throughout the estrous cycle but also throughout pregnancy (Ginther et al., 1996). However, owing to high levels of ovarian and placental progestagens suppressing FSH in the last month of pregnancy, these waves are suppressed (Ginther et al., 1996). At calving, both P4 and E2 levels plummet, FSH levels re-bound and follicular wave emergence commences with a dominant follicle produced, typically 7 to 10 days postpartum (Crowe et al., 1998). As in the follicular phase, E2 is secreted from the dominant follicle but estrous behavior is typically not expressed as there is a requirement for prior P4 exposure in the brain to increase concentrations of E2 receptors in the ventromedial hypothalamus and to trigger estrous behavior (so-called 'P4 priming') (Caraty and Skinner, 1999). Consequently, if this dominant follicle does ovulate it is termed a 'silent 'estrus as it is not associated with behavioral signs (Crowe, 2008). Whether or not the first dominant follicle postpartum ovulates is dependent on whether it produces enough E2 to induce an LH surge and thus induce ovulation. The amount of E2 secreted by the dominant follicle hinges on the size of the dominant follicle, the LH pulse frequency during the dominance phase and the amount of free IGF-I in the dominant follicle (Austin et al., 2001; Canty et al., 2006). Both LH pulse frequency and circulating IGF-I are the critical elements to prompt return to cyclicity (Crowe et al., 2014). In the case of dairy cows, the first dominant follicles ovulate in 30–80% of cows, undergoes atresia in 15–60% of cows and 1–5% become cystic (Savio et al. 1990; Beam and Butler, 1997; Sartori et al., 2004). Anovulation with follicle growth not advancing beyond emergence as been described also (Wiltbank et al., 2002), however, it is much less common than anovulation due to atresia or cystic development of the dominant follicle and further parts of this review will only focus on the two latter.

For those cows that do ovulate, it is relatively common (~70%) for them to experience a shorter than normal luteal phase (~8-10 days) and return to estrus and ovulation 9-11 days after the first 'silent' ovulation (Crowe, 2008). It is understood that this short luteal phase is the result of a premature PGF2α release due to E2 and oxytocin receptor induction by increased E2 secreted by the dominant follicle of the first post-ovulatory wave (Zollers et al., 1993). The probability of whether these short cycles occur is also dependent on when normal ovarian activity resumes (Crowe et al., 2014). In block calving dairy systems, where it is critical that cows re-conceive in timely manner (i.e., by an average by 83 days in milk (**DIM**) to maintain 365 calving interval), any factors that hinders the resumption of cyclicity postpartum are key to understand.

1.2.6 Aberrations in the resumption of cyclicity in the dairy cow; Anovulation and cystic ovarian disease in the dairy cow

Improved fertility following AI of cows that have displayed one or more estruses before the start of the breeding period versus those that have not, is well recognized (Rhodes et al., 2003). As well as anovulation, prolonged luteal phases mostly related to uterine disease are the other important physiological mechanism leading to unobserved estrus in the pre-breeding period (Crowe, 2008). The main factors influencing anovulation are pre- and post- partum nutrition and negative energy balance, parity, season and periparturient disorders or disease (e.g., dystocia, metritis, endometritis, mastitis, ketosis, lameness etc.). Many of the factors that have been linked to anovulation are related either direct or indirectly to a reduction LH pulse frequency (Gilbert, 2016). Many of the key metabolic drivers, such as glucose (McDougall et al., 1993), insulin (Gong et al., 2002) and IGF-1 (Roberts et al., 1997; Patton et al., 2007) have a positive association with an early resumption of cyclicity. Hypoglycemia is well documented as directly suppressing GnRH/LH pulse frequency through sensors in the lower brain stem (Murahashi et al., 1996) but hypoglycemia has indirect impact too as it is the main metabolic fuel of the ovary (Rabiee et al., 1997) . Insulin is key to ovarian steroidogenesis with receptors that are distributed in granulosa, thecal and stromal cells of the ovary (Poretsky and Kalin, 1987). Therefore, high insulin has an indirect effect on LH pulse frequency as it increases dominant follicle production of E2 improving the likelihood of a LH surge and ovulation (Butler et al., 2004). IGF-I too has similar indirect effects as it enhances granulosa cell growth (Hamilton et al., 1999) and alongside FSH increases aromatase activity and thus E2 production by the dominant follicle (Adashi et al., 1985), again augmenting the likelihood of ovulation of the first dominant follicle.

The average duration of calving to first ovulation in dairy cows varies considerably between different sources but tends to be shorter in cows based in indoor [19-22 DIM; (Fonseca et al., 1983)] versus pasture based [43 DIM (McDougall et al. 1995)] dairy systems). However, there is most likely a large within and between herd variation in both systems and between countries. In indoor systems, dairy cows that have not ovulated by 44 DIM are considered to have a prolonged resumption of cyclicity (Lamming and Darwash, 1998). More recent research from New Zealand would indicate that seasonal calving pasture-based dairy cows of higher genetic merit for fertility have shorter calving to first ovulation than those of inferior genetics [41 days for high genetic merit cows versus 60 days for low genetic merit cows (Meier et al., 2021)]. However, this was not found to be the case in an Irish context [24.9 days for high genetic merit cows versus 26.8 days for low genetic merit cows (O'Sullivan et al., 2020)]. However, the latter study does seem to indicate that the duration of calving to first ovulation does appear to be shorter in Irish dairy cows versus New Zealand and may be management related.

Due to the nature of block calving systems, anestrus is often classified as cows who have not ovulated before MSD (Rhodes et al., 2003) and as such could be bias by the particular calving pattern in a research sample. However anestrus has been quantified by Rhodes et al.(2003) who estimated that somewhere between 11 and 38% of dairy cows in indoor systems and 13 and 48% of dairy cows in pasture based systems as anestrus and as such, is a significant issue in both indoor and pasture-based systems.

Many of the individual clinical treatments for anovulation either rely on P4 (intravaginal) synchronization regimes, gonadotrophins synchronization regimes (i.e., Ovsynch and its' variants) or indeed a hybrid of both (Rhodes et al., 2003). Generally, those that include P4 work by a so-called 'pituitary rebound' effect that occurs after the removal of exogenous P4 supplementation, and they tend to have more positive outcomes in terms of estrous expression and the probability of pregnancy (Bisinotto et al., 2015). In seasonal systems, the P4 protocols also have the additional advantage of being much shorter from initiation of the protocol to AI (~10 days) opposed to gonadotrophin based protocols (Double Ovsynch ~27 days; Presynch Ovsynch ~34 days) while still having favorable results (Rhodes et. al., 2003; Herlihy et al., 2011). Although hormonal treatment plays an important part in addressing anovulation in an individual cow, nutritional management strategies play an important role in the prevention of anovulation and overall reproductive performance in the herd. In pasture-based herds it is now well established that calving cows at an optimal BCS [3.0-3.25 in Ireland (Buckley et al. 2007); 5.0-5.5 in New Zealand (Roche et al., 2007)] and minimizing postpartum BCS loss [≤0.5 BCS units in Ireland (Buckley, Dillon et al. 2007)]; 1.5 units in New Zealand (Roche et al. 2007)] are key drivers for reducing anovulation incidence and improving overall reproductive performance (Butler, 2014).

In cows, the development of cystic ovarian disease is a sequela of the resumption of cyclicity postpartum. Clinically, it has been defined as a follicle greater than 25mm that persists for greater than 10 days with no corpus luteum present on either ovary (Day, 1991). More modern definitions of cystic ovarian disease recognize that cysts are dynamic structures both physically and endocrinologically and they may not require a 10-day period of persistence to be classed as such (Gilbert, 2016). They are most often diagnosed by transrectal ultrasonography and present most commonly as singular cysts of a large size (typically >25mm) although can be multiple cysts can appear too (Gilbert, 2016). They can be subclassified further based on their appearance on ultrasound. Those cysts with a thin wall (<3mm) that is unluteinized are termed 'follicular cysts' while those that are thick walled (>3mm) and are luteinized are termed 'luteal cysts'(Garverick, 1997). Anestrus is the most common sign and therefore cystic cows are indistinguishable to the farmer from other cows that are suffering from anovulation in the postpartum (Day, 1991). However, if a follicular cystic follicle is E2 secreting, then the farmer may note nymphomania (i.e., persistent signs of estrus) (Day, 1991). Luteal cysts always present with anestrus due to P4 secretion and the negative feedback this exerts on the hypothalamic-pituitary axis (Day, 1991).

The incidence of cystic ovarian disease is reportedly 5-30% (Garverick, 1997) but this depends on the time frame postpartum and the population of cows that has been studied (Bartlett et al., 1986). In an

Irish seasonal dairy context, Mee et al. (2009) has found that the prevalence of cows with cystic ovarian follicles was 3.9% when cows were examined in the pre-breeding period at an average of 52 DIM. A much higher incidence is observed in earlier lactation (Bartlett et al., 1986), in high yielding dairy cows (Ashmawy et al., 1992), those cows with a high degree of NEB (Vanholder et al., 2006) and in cows with uterine inflammation (Bosu and Peter, 1987) or other disease processes such as lameness (Melendez et al., 2003). It may also be higher in certain herds due to genetic predisposition to development and its correlation with milk production traits (Hooijer et al., 2001).

Like anovulation much of the pathogenesis is linked to direct or indirectly effects on LH pulse frequency (Gilbert, 2016). Typically, one pulse per hour required is required to induce ovulation (Forde et al., 2011) and if this is not achieved, the dominant follicle will not ovulate and may persist and grow into cystic structure. Many underlying physiological mechanisms have been explored to explain how some of these aforementioned stresses on cows result in inadequate LH pulse frequency and the development of cystic follicles (Vanholderr et al., 2006). These include: failure of sufficient E2 production from dominant follicle, low free IGF-1 (due to higher IGFBP4 and IGFR1 receptors in cystic follicles), hypothalamic insensitivity to positive feedback from E2 or stress inhibition of the hypothalamic responsiveness (Vanholder et al., 2006), increased cytokine expression (IL-6, IL-8 and TNFalpha) (Stassi et al., 2017) and sub-luteal progesterone levels altering LH pulses leading to follicle persistence (Kinder et al., 1996).

Many cows with cystic ovarian disease will self-resolve over time, especially when they develop in early lactation (Garverick, 1997; Sakaguchi et al., 2006). Manual rupture of the cystic structure has been discussed in the past but is now contraindicated due to likely trauma, hemorrhage and possible adhesions that may result from it (Nanda et al., 1991). Follicular aspiration with an ovum pick-up device has been investigated (Lievaart et al., 2006) but its overall efficacy in resolving cysts is not well documented. Administration of GnRH to follicular cysts to induce ovulation or luteinization of another responsive dominant follicle is what has been most widely researched and advocated (Gilbert, 2016). PGF2α should be given to cows with luteal cysts to induce luteolysis of an already luteinized follicle and the resumption of cyclicity (Gilbert, 2016). Like anestrus cows, P4 intravaginal devices for a period of 7-8 days with PGF2α are advocated (Gümen and Wiltbank, 2005). As with many interlinked reproductive diseases, prevention of predisposing risk factors and treatment of concurrent disease is likely to ensure reduced incidence and enhanced response to therapeutics, respectively.

1.2.7 Physiological recovery of the reproductive tract in the dairy cow

Optimal future fertility in the dairy cow depends on numerous postpartum physiological events occurring in synchrony and without issue. The key events that must occur are as follows; expulsion of the fetal membranes, uterine involution, endometrial restoration, pathogenic bacterial elimination from the reproductive tract and of course, resumption of ovarian cyclicity as previously discussed. Many of

these events are interlinked and disruption of one may hamper or delay the resolution of the other and vice versa.

Most dairy cows will expel their fetal membranes by 6-8 hours postpartum, but some may take 24 hours to do so (Gilbert, 2016). For this reason, there is some controversy over when the condition becomes pathological [i.e., retained fetal membranes (RFM)] versus normal biological variation among cows. However, it is generally accepted that if fetal membranes are retained by 24 hours postpartum it is considered abnormal (Beagley et al., 2010). The interdigitation between the fetal and maternal villi in the caruncular areas of the placentome is secured by adhesive proteins, largely collagen (Eiler and Hopkins, 1993), cellular attachments and physical arrangement of the cotyledons and the caruncle (Beagley et al., 2010). For appropriate detachment and expulsion to occur in a timely manner postpartum a complex sequence of pre-partum physiological events must take place to breakdown these attachments (Beagley et al., 2010). These are triggered by the rise in fetal cortisol near term that switches placental steroids synthesis away from P4 and towards E2 production (Flint et al., 1979). The decrease in P4 removes the myometrial block on contractility and E2 promotes oxytocin receptors and PGF2α release, further increasing myometrial activity (Fuchs et al., 1999). This PGF2α also causes luteolysis, further decreasing circulating P4 as production from the CL dwindles (Musah et al., 1987). Binucleate giant cells, which secrete PGE2, decrease in the pre-partum period with a ratio of low PGE2 to high PGF2α recognised as an important part of normal placenta detachment (Wischral et al., 2001). A relaxin-like hormone (insulin like peptide 3) is also released from the CL at this time and it promotes relaxation of the genital tract during parturition, though its role in placental detachment remains less clear (Noake et al., 2018).

Whatever the case these hormonal changes, initiate vascular changes and uterine contractility which are recognized as essential elements of placental expulsion (Laven and Peters, 1996). However, arguably more importantly, this hormonal shift stimulates collagenase and other enzyme (e.g., hyaluronidase, Nagase) activity which degrades the collagen and proteoglycans at the foeto-maternal interface something which is vital for detachment (Kankofer et al. 2000; Beagley et al., 2010). Additionally, the role that the immune system plays in detachment is well recognized with neutrophil, macrophage and lymphocyte invasion of the placenta (Gunnink, 1984) , cytokine production (Heuwieser and Grunert, 1987) and major histocompatibility complex class I antigens noted as key for placental detachment (Davies et al., 2004). Indeed, hormonal aberrations [e.g., low circulating E2 (Wischral et al., 2001) and high PGE: low PGF ratios (Slama et al., 1994)], disrupted enzymatic activity (e.g., in the case of exogenous steroid induction of parturition) and depression in immune function (Kimura et al., 2002) are well understood to be the physiological basis for RFM.

Postpartum involution is defined as the physical reduction in size of the formerly gravid and non-gravid uterine horns and cervix to their pre-conception size (Gier and Marion,1968). Normal uterine involution in both horns occurs on a logarithmic scale in the first fortnight postpartum with a gradual decrease in

diameter thereafter (Gier and Marion, 1968; Sheldon et al., 2003). By one-month postpartum, there is no perceptible difference in uterine diameter between gravid and non-gravid horns and most authors expect that it is complete before 42 days postpartum (Sheldon, 2004). Much of the involution of the genital tract is brought about by cellular shrinkage and atrophy of smooth muscle, reduction in collagen and expulsion of the remaining fetal fluid, blood, membranes and sloughed caruncles (collectively referred to as 'lochia') (Sheldon, 2004). Hormonally, prostaglandins play a role in involution with elevated levels of PGF2α metabolites postpartum being associated with decreasing uterine horn diameters (Eley et al., 1981) but this is because they are involved in tissue remodeling rather than being a direct trigger for involution (Sheldon, 2004). This likely explains why exogenous administration of PGF2α and other related hormones (e.g., oxytocin and E2) have shown little or equivocal benefit in hastening involution (Tian and Noakes, 1991).

The complex interplay between the ovary and the uterine activity is probably none more evident than in the early postpartum period. Studies that have shown that there is greater folliculogenesis on the ovary contralateral to the previously gravid horn (Nation et al., 1999). Additionally, these follicles in the ovary opposite to the previous gravid horn grow larger, produce more E2 and have a greater probability of ovulation as a dominant follicle (Spicer et al. 1986; Kamimura et al. 1993; Nation et al., 1999). Contrary to what one may think, selection of the first postpartum dominant follicle on the ovary on the same side of the gravid horn is a marker of subsequent fertility (Bridges et al., 2000; Sheldon et al., 2000). It is postulated that follicular wave emergence and selection of a dominant follicle on the ovary ipsilateral to the gravid horn is facilitated in the absence of uterine disease (Sheldon, 2004). Indeed, many postpartum disorders (e.g., dystocia, hypocalcemia, retained placenta, metritis, and endometritis) have been associated with delayed uterine involution (Sheldon, 2004).

Once the fetal placenta is expelled postpartum and simultaneous to the process of uterine involution, the endometrium of the cow begins to regenerate. This commences with the necrosis and sloughing of the stratum compactum of the caruncles, which then undergo liquefaction and are eliminated as part of the lochia as involution rapidly progresses in the first week postpartum (Gier and Marion, 1968). This lochial discharge tends to be a non-fetid, mucoid material that is yellow, brown or red in colour with volumes of up 1 to 2 liters being expelled (Sheldon, 2004). Once the caruncles are sloughed, the smooth surface of the stratum compactum is re-epithelialized starting at the edge of the caruncle (Wagner and Hansel, 1969). Repair of these tissues requires leukocyte migration and accumulation for tissue repair and bacterial suppression and this is regulated via various chemotactic mediators. However, cows that have impaired immune systems due to disease (e.g., NEB) may not be able to as effectively mount an immune response thus prolonging the time required for uterine recovery (Wathes et al., 2009). In healthy cows without underlying disease or who did not have excess tissue trauma at birth, luminal epithelium of the endometrium takes approximately 20 to 30 days to fully repair (Wagner and Hansel, 1969).

Nowadays it is widely recognized that the bovine uterus is not sterile environment and has its' own microbiome throughout life including during pregnancy (Karstrup et al. 2017; Moore et al., 2017). However, the relaxation of vulva and cervix at calving and for a brief period afterwards, offers the opportunity for gross contamination with environmental bacteria of varying levels of pathogenicity while the hormonal changes (notably a rise in glucocorticoids) assist in a relative bloom in the resident microbiota from the previous pregnancy (Moore et al., 2019). This microbial contamination can be detected by simple culture in nearly all dairy cows [~93% (Marion et al., 1968)] in the first fortnight postpartum but it is only considered pathogenic if the majority of bacteria are not eliminated after 3-4 weeks (Gilbert et al. 2005; Sheldon et al., 2006a). As with most infectious disease, the fine balance between infectious pressure (i.e., an infectious agent's environmental abundance and pathogenicity) and the host's immunity (i.e., innate and acquired responses) are key to whether reproductive tract infections establish and the severity of such infections.

It is important to stress that this balance may lead to fluctuations in bacterial load due spontaneous contamination, clearance and recontamination with numbers often increasing between day 7 and day 14 post-partum indicating that contamination at calving is not the sole etiology of reproductive tract infections (Griffin et al., 1974). The most commonly isolated bacteria are *Escherichia coli, Trueperella (Arcanobacterius) pyogenes, Fusobacterium necrophorum, Prevotella* spp*, Bacillus licheniformis* and *Streptococci* spp (Sheldon, 2004). The first four listed species ae the most commonly associated with reproductive tract infections and indeed, there is a recognized synergy between *T. pyogenes, F. necrophorum*, and *Prevotella* via the production of leukotoxins and growth factors (Sheldon, 2004).

Although the effect of uterine infection and inflammation seems clear, the effect of these infections on the ovary are less obvious but no less profound. Cows with uterine infections have slower growth of the dominant follicle, lower circulating E2 concentrations, and lower probability of ovulation (Sheldon et al., 2002b). This is believed to be mediated through passage of host cytokines (e.g., interleukin-6 and tumor necrosis factor alpha) and bacterial lipopolysaccharide to the ovary via the counter current exchange system where they disrupt ovarian steroidogenesis and follicular growth (Spicer, 1998; Herath et al., 2007). Conversely, the hormonal environment also influences the likelihood of bacterial infection. Estrus and an E2 environment facilitate elimination of bacteria through increased contractility and lack of an immunosuppressive effect (Sheldon, 2004). P4 has a quiescent effect on the myometrium and an immunosuppressive effect which facilitates bacterial proliferation (Noakes et al., 1990). Once infection is established in the uterus, it will inhibit PGF2α release and luteolysis resulting in pyometra and abnormal or prolonged luteal phases (Noakes et al., 1990; Opsomer et al., 2000).

Rapid elimination of bacteria also depends on the host's immune system response of which there are two integral parts; the innate (i.e., non-specific primary defense barriers) and the acquired (i.e., specific secondary learned defense barriers) systems (Sheldon, 2004). As many uterine infections are polymicrobial, the innate immune system is the main system of defense while the role of adaptive

immunity in bacterial elimination is less clear (Sheldon, 2004), yet the fact that research into a metritis vaccine showed some improvement in clearance of infection would suggests it also has a role to play (Machado et al. 2014).

The innate immune system of the reproductive tract consists of the physical, physiological and inflammatory defenses. The main physical defenses include the vulva, vestibule, vagina, cervix and an intact vaginal and endometrial epithelium all of which prevent the entry and establishment of infection, while any damage to them as result of dystocia weakening these defenses (Sheldon 2014). The physiological defenses include the production of epithelial production of mucous, lysozymes, antimicrobial peptides and glycoproteins which prevent bacteria from reaching the plasma membrane (Sheldon, 2004). The main inflammatory defenses include chemokine and cytokine signaling in response to bacteria and/or cellular damage, stimulation of the acute phase proteins response (e.g., α1-acid glycoprotein, haptoglobin and ceruloplasmin), complement system activation and influx of phagocytic leukocytes (Sheldon, 2014). Response to bacteria and endotoxins is mediated via the toll-like receptors on leukocytes, epithelial and stromal cells which result in the secretion of signaling molecules such as interleukins (e.g., IL-1β, IL-6, IL-8), tumor necrosis factors and PGE2 (Sheldon, 2014). These increase vascular permeability, stimulate the local and systemic inflammatory response, and create positive feedback which further augments neutrophil migration and activation (Sheldon, 2014). Such inflammatory responses can result in severe clinical signs such as marked pyrexia, depression and shock and thus there is a careful balance to limit inflammation to meet the threat while also ensuring pathogens are eliminated (Sheldon, 2014).

Disruption in any of the physical, physiological and inflammatory defenses of the innate immune system results in the establishment and persistence of infection and the local and systemic inflammatory response to this is what manifests as the postpartum clinical diseases of the reproductive tract that have profound effects on productivity and fertility in the modern dairy cow.

1.2.8 Aberrations in the physiological recovery of the reproductive tract in the dairy cow; Reproductive tract infection, inflammation and disease

Reproductive tract infections or disease are clinically described and defined as oophoritis, salpingitis, metritis, endometritis, cervicitis and vaginitis. Both metritis and endometritis are the most researched and of the greatest economic importance in the modern dairy cow (Machado and Bicalho, 2018) and these will be discussed in further detail in this section. The key definitions, diagnostic methods, risk factors for development, likely impacts on fertility and production as well as current proposed therapies are outlined in Table 1.2. The challenges associated with some of the existing definitions and diagnostics are outlined in section 1.2.9.

Metritis is the defined as inflammation of the entire uterus (i.e., endometrium, myometrium, and perimetrium) and is caused predominantly by a polymicrobial bacterial infection (most notably due to a

mix of *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum*) and can be further sub-classified as either puerperal or clinical based on its clinical presentation (Sheldon et al., 2006a). Puerperal metritis is classified as a cow that presents within the first three weeks postpartum (typically first within 10 days) with an enlarged uterus filled with a fetid watery red-brown uterine discharge, associated systemic signs (depressed milk production/dullness) and a pyrexia (>39.5 °C) (Sheldon et al., 2006a). Those cows that are not systemically unwell but have an enlarged uterus and a purulent or fetid discharge and still within the first three weeks postpartum are classified as having clinical metritis (Sheldon et al., 2006a). Metritis has detrimental effects on productivity by decreasing milk production, subsequent reproductive performance and increasing the risk of culling (Dubuc et al. 2011; Wittrock et al. 2011; Giuliodori et al., 2013b). Cows with metritis are also at greater risk of developing endometritis in later lactation (Dubuc et al., 2010b). Interestingly, some authors have suggested that for multiparous cows with metritis the risk of being culled is more related to their negative production rather than reproduction effects (Wittrock et al., 2011). Puerperal metritis tends to be treated with systemic antibiotics, oral or intravenous fluids non-steroidal anti-inflammatory therapy and uterine lavage or siphoning (Gilbert, 2016). Although, a positive effect with some of the commonly used antibiotics (e.g., ceftiofur, ampicillin) has been noted (Haimerl et al., 2017), there is very little research evidence to show a positive effect of the aforementioned supportive therapies (Haimerl and Heuwieser, 2014), although intuitively they make sense, particularly fluid therapy and non-steroidal anti-inflammatories.

Endometritis is defined as superficial inflammation of the endometrium, extending no deeper than the stratum spongiosum (Bondurant, 1999). Endometritis is caused predominantly by a mixed infection of the bacterial pathogens (i.e., *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum*) although other more specific pathogens have been noted in specific infections (e.g., *Brucella abortus*, *Leptospira* spp., *Campylobacter fetus venerealis*, *Tritrichomonas foetus*, *Mycoplasma bovis* and Bovine *herpesvirus 4*) (Noakes et al., 2018). Endometritis is also sub-classified to as either clinical or subclinical endometritis (Sheldon et al., 2006a). Clinical endometritis is classified as the presence of purulent material (>50% pus) in the vagina 21 days post-partum or mucuopurulent (approximately 50% pus, 50% mucus) in the vagina 26 days post-partum with the absence of systemic signs (Sheldon et al., 2006a). Subclinical endometritis is classified as the presence of greater than 18% polymorphonuclear leukocytes (**PMNs**) in uterine cytology sample from cows between 21–33 DIM or >10% PMNs at 34–47 DIM (Sheldon et al., 2006a). Both classifications are only considered subclinical endometritis in the absence of clinical endometritis (i.e., mucopurulent discharge) (Sheldon et al., 2006a). More recently and based on a systematic review of the literature (de Boer et al., 2014), a lower threshold of >5% between 35-40 DIM has been suggested as it is associated with impaired reproductive performance. Indeed, as different diagnostic methods (see Table 1.2) and methods to determine the threshold for positivity for each condition have evolved, some of the traditional definitions and the thresholds for positivity have altered and evolved alongside the research. These challenges will be reviewed further in the next section (1.2.9).

Clinical and subclinical endometritis have no systemic effects on the cow and thus, do not tend to limit milk production (Machado and Bicalho, 2018). Clinical endometritis tends to have a high clinical self-cure rate, meaning that the further into lactation a cow progresses the more likely that she will be to resolve the purulent discharge themselves with no treatment (Gautam et al. 2010; Giuliodori et al., 2017). Some publications report clinical self-cure rates ranging from 57 to 77% (Dubuc et al., 2010b; Gautam et al., 2010; Giuliodori et al., 2013a; Giuliodori et al., 2017). This self-cure may be a true complete cure or a change to the subclinical state and factors that influence this include DIM at the first diagnosis and the severity of the discharge (i.e., those with more purulent material having lower odds of self-cure or medical cure following treatment) (Burke et al., 2010; Giuliodori et al., 2017). However, even though both clinical and subclinical endometritis have limited systemic effects on the cow and production, they are both economically important diseases as they have lasting negative effects on subsequent reproductive performance (McDougall et al., 2007) and as a result, have a major negative financial impact on the modern dairy industry.

Medical treatment for clinical endometritis has long focused on the use of licensed local intrauterine antibiotics [e.g., cephapirin (Metricure®, MSD Animal Health)], the exogenous administration of PGF2α (Lefebvre and Stock, 2012) and some alternative intrauterine therapies (e.g., hypertonic dextrose, enzymes) (Drillich et al. 2005; Machado et al. 2012). However, only intrauterine cephapirin has some convincing evidence to suggest it improves reproductive performance post treatment (particularly in seasonal calving systems) (Runciman et al. 2008; McDougall et al. 2013) and anecdotally, is widely used in Ireland for this purpose. Intuitively, exogenous PGF2α administration in the presence of a CL will result in luteolysis and estrus induction and switch from an immunosuppressive P4 environment to an immunoreactive E2 environment while also stimulating uterine contractions yet applied research has not supported this physiological logic (Lefebvre and Stock, 2012; Gilbert 2016). Treatment advocated for subclinical endometritis is similar to clinical endometritis (i.e., intrauterine antibiotics and exogenous PGF2α) but the efficacy of such treatments remains even more controversial (Wagener et al., 2017).

The development of reproductive tract disease, like many diseases relates to an interaction between the host (i.e., the cow), the environment (i.e., the dry, calving, early lactation locations) and the pathogens (Machado and Bicalho, 2018). This means that if the bacterial load is high even quite immunologically competent cows will succumb to infection or indeed, the converse is possible that even when bacterial loads are low, cows may still succumb to disease if they are immunologically incompetent. Research on risk factors within each of these three categories helps understand disease development and it informs methods to help in targeting prevention or diagnosis. Host or cow-level risk factors have been the most widely studied and relate to factors resulting in tissue trauma or impaired immunity due to metabolic issues. Environmental risk factors have been less widely studied and have had more equivocal result, and relate to factors related to poor hygiene of the dry, calving or early lactational environment. Pathogen associated risk factors studied include pathogenic factors such as, *FimH* and pyolysin that

assist in the developmental of reproductive tract disease (Sheldon et al. 2009). The main cow and environmental risk factors for the development of all the reproductive tract diseases discussed are outlined in Table 1.3.

Considering there are costs associated with both the diagnosis and treatment of reproductive tract disease to the farmer, further research into the risk factors and additional diagnostics tests is warranted. This research would assist farmers and veterinarians in targeted sampling or even in targeted preventative strategies for cows that might be at risk of reproductive tract diseases.

Table 1.2. Summary of the main disorders/diseases of reproductive tract recovery, their definitions, common diagnostic methods used, risk factors, impacts on fertility/production and proposed therapeutic options. References are indicated by number in footnotes.

Main disorders	**Definitions[1, 2, 4]**	**Common Diagnostic Methods Used [1, 2]**	**Proposed Risk Factors for Disease[1, 2, 3 ,4]**	**Impact on fertility and production[3]**	**Proposed Therapeutic Options[4]**
Retained Fetal Membranes (RFM)	Failure to expel fetal membranes after either 12-or 24 hours.	Direct observation of retained membranes. OR Gloved vaginal/cervical/uterine examination to note the retained membranes.	Induced parturition Shortened gestation Abortion/placentitis Twinning Dystocia/Fetotomy/Caesarean Hypocalcemia Vitamin E Selenium deficiency	Not directly associated with impaired subsequent fertility. Cow that subsequently develop metritis with systemic effects will likely have impaired milk production. Cow that subsequently develop endometritis will likely have impaired subsequent fertility.	No treatment provided systemically well. Treatment with systemic antibiotics, anti-inflammatories +/- fluids only if systemic signs present. Manual removal provided it is atraumatic. Ecbolic agents (e.g. oxytocin or PGF2α) are rarely useful.
Metritis	**Subclassified into;** *Puerperal metritis* = Cow with an enlarged uterus, fetid watery red-brown uterine discharge and signs of systemic illness <21 DIM but typical present in first 10 days. *Clinical metritis* = Cow with an enlarged uterus, purulent/fetid watery uterine discharge but no systemic signs < 21DIM.	Full clinical examination. Gloved vaginal/cervical/uterine examination.	Induced parturition Prolonged gestation RFM & Dystocia Parity & Breed Stillbirth Twining LDA, NEB & ketosis Elevated Haptoglobin	Cow with metritis will have impaired milk production in early lactation. Cow that subsequently develop endometritis will likely have impaired subsequent fertility.	Treatment with systemic antibiotics, anti-inflammatories +/- fluids only if systemic signs present. No treatment provided systemically well. Uterine lavage with physiological saline provided cautiously undertaken. Ecbolic agents are rarely useful.

Main disorders	**Definition**[1, 2, 5, 6, 8]	**Common Diagnostic Methods Used**[1, 2, 5, 7, 11]	**Proposed Risk Factors for Disease**[1, 2, 3 ,4, 9, 10, 12]	**Impact on fertility and production**[3]	**Proposed Therapeutic Options**[4]
Clinical Endometritis (NB. Many now be refer to as purulent vaginal discharge (**PVD**) depending on the method of diagnosis used)	Purulent (>50% pus) discharge in the vagina at ≥ 21 DIM OR Mucopurulent (approximately 50% pus, 50% mucus) discharge in the vagina at ≥ 26 DIM Both are not associated with systemic illness Flecks of purulent material have been shown to impair reproductive performance and can be considered a positive case. Graded on a scale of 0-3 or 0-5 (see Table 1.3)	Direct visual observation of purulent discharge Direct visual assessment of vaginal/cervical discharge with vaginoscopy. Indirect visual assessment of vaginal discharge following retrieval with a gloved hand or Metricheck® device. Indirect of visual assessment of uterine content with transrectal ultrasound.	RFM Metritis Twinning Dystocia Stillbirth Male Calf Urovagina NEB, ketosis & BCS loss Elevated Haptoglobin Hypocalcemia Genetics	Significant impairment of subsequent fertility. Not associated with milk production losses.	No treatment and may note an apparent or true so-called 'self-cure' Administration of intrauterine antibiotics (e.g. cephapirin) Administration of PGF2α to induce luetolysis and estrus in those cows with a corpus luteum but efficacy is controversial
Subclinical endometritis	>18% polymorphonuclear leukocytes (PMNs) in uterine cytology sample from cows between 21–33 DIM OR >5-10% neutrophils at 35–40 DIM[5] Both should be diagnosed in the absence of clinical endometritis.	Cytological assessment under microscope after attained sample via cytobrush/uterine lavage. Biochemical tests (such as the leukocyte esterase test) to detect the present of leukocytes Histopathological assessment of uterine biopies	Metritis Parity Low calving BCS NEB & ketosis Elevated Haptoglobin Bedding material Milk production	Significant impairment with subsequent fertility. No associated with milk production losses	No treatment as efficacy is controversial for those listed below. Administration of intrauterine antibiotics (e.g. cephapirin). Administration of PGF2α to those cows with a corpus luteum.

Table 1.2 references= [1] (Gilbert, 2016), [2](Sheldon et al., 2006a), [3](Machado and Bicalho, 2018), [4](Sheldon, 2019), [5](de Boer et al., 2014), [6](McDougall et al., 2007), [7](Mee et al., 2009).[8](Dubuc et al., 2010a), [9](Dubuc et al., 2010b), [10](Cheong et al., 2011), [11](Barlund et al., 2008), [12](Gautam and Nakao, 2009).

1.2.9 Challenges around the definitions and diagnosis of reproductive tract disease beyond 3 weeks post-partum and the research requirements

Although Sheldon et al., (2006a) aimed to define the conditions (i.e., clinical and subclinical endometritis), the explosion of research into different diagnostic methods and establishment of various thresholds for positivity have resulted in large amount of variability in the literature related to definitions, diagnostics and thresholds for positivity of reproductive tract disease diagnosed beyond three weeks postpartum. This challenge was well highlighted by some of the leading authors in the field when they conducted a systematic review of the literature (de Boer et al., 2014). A brief history in relation to these challenges in diagnostic definitions and test assessment for reproductive tract disease beyond three weeks pos-partum is therefore necessary to review.

Sheldon and colleague's original definition and diagnostic method to describe clinical endometritis involved both vaginal and cervical examination with a gloved hand or speculum by a trained professional to assess both the type of vaginal discharge and estimate the cervical diameter by direct observation and/or palpation (Sheldon et al., 2006a). Cervical diameter greater than 7.5cm and mucopurulent discharge from the cervical os were what was defined as clinical endometritis (Sheldon et al., 2006a). However, this diagnostic method was more time consuming and not as practical, particularly in seasonal systems where large numbers of cows are potentially examined in a single visit prior to the breeding season. This led to the development of a stainless steel rod with a rubber hemisphere attached at the end called the Metricheck® device in countries with seasonal calving dairy cows like New Zealand [Simcrotech, Hamilton, New Zealand, (McDougall et al., 2007)]. This device is inserted through a cleaned vulva and then into the cranial vagina until resistance is met at the cervical os and then it is slightly elevated, and the device is withdrawn caudally (McDougall et al. 2007). The material within the rubber hemisphere is then graded on a scale of 0 to 5 (McDougall et al., 2007) which differed from the original 0 to 3 scale that was described by Sheldon's team (Williams et al., 2005). The differences in grading scales and cases definitions is highlighted in Table 1.3. Despite this method not assessing the vagina or cervix visually or with palpation, it was found to be more sensitive in detecting clinical endometritis than vaginoscopy (McDougall et al., 2007).

Table 1.3. Comparison of the scoring tables for clinical endometritis or purulent vaginal discharge (PVD). Based on the two studies in the table the scores that were deemed positive cases for disease are outlined.

Score number	**Williams et al. (2005)**	**Positive Case**	**Score number**	**McDougall et al. (2007)**	**Positive Case**
0	Clear or translucent mucus; .	No	0	No discharge	No
			1	Clear mucous discharge	No
1	Mucus containing flecks of white or off-white pus	No	2	Flecks of purulent material within otherwise clear mucus,	Yes
2	Less than 50 mL exudate containing ≤50% white or off-white mucopurulent material.	Yes	3	Mucopurulent but <50% purulent material	Yes
3	50 mL exudate containing purulent material, usually white or yellow, but occasionally sanguineous. (Assessment of odour was also performed)	Yes	4	Mucopurulent with >50% purulent material	Yes
			5	Mucopurulent with >50% purulent material and with an odour	Yes

Further work by Dubuc et al. (2010a), suggested that the term clinical endometritis was not the most appropriate definition to describe the assessment of vaginal discharge as a proxy for clinical endometritis. The authors suggested the term purulent vaginal discharge (**PVD**) would be more appropriate and descriptive because this diagnostic method (especially when performed with a Metricheck® device) may also include discharge originating from a cranial vaginitis or cervicitis in addition to a clinical endometritis.

Indeed, both vaginitis and cervicitis are poorly defined inflammatory conditions of the dairy cow with a limited number of authors attempting to fit diagnostic criteria to them or assess their effect on subsequent reproductive performance (Rahim Ahmadi et al. 2006; Deguillaume et al., 2012). However, Deguillaume et al. (2012) did established that endocervical inflammation of greater than 5% neutrophils in a cytological sample taken before 35 days postpartum was detrimental to subsequent fertility. This study also suggested a cumulatively negative impact on subsequent fertility when both endocervical and endometrial inflammation occurred together. It is therefore likely that with the current commonly used method (i.e., Metricheck® device) as it only assesses retrieved vaginal discharge that it is including both vaginitis and cervicitis as well as clinical endometritis. Consequently, the suggested definition of PVD by Dubuc et al., (2010a), was deemed by many as the more appropriate description and explains why it is now a more commonly used term among researchers than clinical endometritis when only vaginal discharge is visually assessed.

The thresholds for what was deemed a case of clinical endometritis or PVD has also differed as further research has been conducted and this is also presented in Table 1.3. Either a scores of > 1 [≥ flecks of pus, (McDougall et al. 2007; Giuliodori et al., 2017)] or ≥2 [≥ mucopurulent, (LeBlanc et al. 2002; Williams et al., 2005)] have both been defined as positive for disease. One of the issues of determining the appropriate threshold for positivity in the case of any diagnostic test for reproductive tract disease is the absence of a 'gold 'standard reference test and as a result many researchers have relied on uterine cytology or bacteriology or future reproductive outcome as a method to calculate test parameters and decide on the appropriate threshold for disease (de Boer et al., 2014). It has been suggested that the use of reproductive outcomes in future clinical research is the most logical and appropriate way to validate tests (de Boer et al., 2014).

When this method has been adopted, diagnostic test parameters for PVD have been found to have low sensitivity but moderate to high specificity relative to pregnancy status (de Boer et al. 2014). Low test sensitivity results in high false negative diagnosis of disease, in this case, cows with PVD that are diagnosed as not having PVD. Low test specificity results in high false positive diagnosis of disease, in this case, cows without PVD that are diagnosed as having PVD. It was also noted by de Boer et al. (2014) that a diagnostic test for PVD with higher sensitivity (i.e., misses less true PVD cases) and lower specificity (identifies more PVD cases that are in fact false) may be more important in seasonal herds than in year-round herds because missing infected cows at a single pre-breeding examinations is less

possible and time thereafter to conceive is very limited. However, basing test parameters and positivity thresholds on reproductive outcome does pose issues as reproductive outcome is influenced by many parameters and is an event in the future. This was acknowledged by the same authors (de Boer et al., 2014) and they have additionally suggested that enhanced statistical techniques, such as the Bayesian approach for tests assessment in the absence of a gold standard, could be a potential path to improve the understanding of the validity of future and current diagnostic tests (de Boer et al., 2014).

A further complication in the diagnosis of clinical or subclinical endometritis is the advent of transrectal ultrasonography. In some cases of endometritis, it is likely that vaginal discharge may not be present either due to a closed cervix (e.g., in the case of a pyometra) or when there is intermittent discharge from the uterus through the cervix (Šavc et al., 2016). As a result, many have used transrectal ultrasound to assess visible intrauterine fluid, endometrial thickness or horn diameter to assist in diagnosis of endometritis (Mee et al. 2009; Senosy et al. 2009; López-Helguera et al. 2012; Šavc et al., 2016).The advantages of this diagnostic method are obvious in that it can be quickly visually assessed at cow-side as opposed to the more laborious methods of preparing and reading cytological slides. Various definitions in terms of intrauterine fluid volumes that are diagnostic for endometritis using ultrasonography (**UE**) have been established but range from the non-specific presence or absence of fluid (Senosy et al., 2009; Meira et al.; 2012) to scoring the echogenicity as echogenic or anechogenic (López-Helguera et al., 2012) to specific measurements of >1mm (Barlund et al., 2008), >2mm (Mee et al., 2009), >3mm (Kasimanickam et al., 2004; Barlund et al., 2008) of intrauterine fluid. In terms of endometrial thickness values of >7-8mm have been shown to be diagnostic of UE (Barlund et al., 2008). It is likely that ultrasonography for the diagnosis of endometritis lines somewhere in the middle ground between the clinical and subclinical states of endometritis. The agreement between tests has been shown to be moderate to poor. Investigations by some authors have shown that the correlation between PVD (i.e., diagnosed with a Metricheck®) and UE (i.e., diagnosed with intrauterine fluid detection) was found to be moderate [$r = 0.5$; (Senosy et al., 2009)] while the agreement between UE and subclinical was endometritis was fair [$\kappa = 0.28$; (Kasimanickam et al., 2004)].

Like PVD, in the case of UE, determination of the appropriate threshold for disease positivity and the estimation of test parameters has proven challenging in the absence of a gold standard. Relative to pregnancy status, the sensitivity for UE is generally low but the specificity is general high (Barlund et al., 2008). However, when comparing UE to cytological endometritis as diagnosed by cytobrush, test sensitivity for UE is generally higher while the specificity lower (Barlund et al., 2008). However, application of enhanced statistical techniques, as mentioned previously, has the potential to improve the understanding of this diagnostic test also and should be considered in further research assessing this diagnostic test.

In the absence of clinical detectable changes in discharge, subclinical (or cytological) endometritis has been classified based on the proportion of PMNs in a uterine cytology sample. Numerous cut-offs of the

proportion of PMNs have been proposed based on the days postpartum when the sample is taken and its association with subsequent reproductive performance (de Boer et al., 2014). Sheldon et al. (2006a), originally proposed cut-offs of greater than 18% PMNs in samples from cows between 21–33 days postpartum, or greater than 10% PMNs between 34–47 days. However more recently, it has been suggested that greater than 5% PMNs in samples from cows between 35 to 40 days postpartum is a more accurate cut-off as this was more consistently associated with diminished reproductive outcomes (de Boer et al., 2014). Various techniques have been used in the research to obtain an endometrial cytological sample from cytobrushes (Kasimanickam et al., 2004; Dubuc et al. 2010a), uterine lavage (Kasimanickam et al., 2005; Cheong et al., 2011), even cytotype (Pascottini et al., 2015). In addition, standardized cytological slide reading procedures have varied between the research groups (de Boer et al., 2014) with methods of stain preparation and cellular counts to establish the proportion also differing. The correlation coefficient between PVD and subclinical endometritis (i.e., diagnosed with cytobrush) is moderate [r = 0.59; (Senosy et al. 2009)] while the others have found the agreement fair [κ range ~ 0.12-0.3 (Dubuc et al. 2010a; McDougall et al. 2011)]. The agreement between UE and subclinical endometritis (i.e., diagnosed with cytobrush) is fair [κ = 0.28; (Kasimanickam et al. 2004)]. The sensitivity for subclinical endometritis (diagnosed with cytobrush) are generally low but the specificity are general high relative to pregnancy status (Kasimanickam et al. 2004; Dubuc et al., 2010a)] but estimation of these test parameters too suffers from issues in relation to the absence of a gold standard test.

From a practical standpoint, use of a cytobrush or lavage and cytological slide interpretation to diagnose subclinical endometritis on farm has many disadvantages. It does not facilitate a rapid diagnosis, hampering the number of cows that can be examined in a single visit which is very relevant to seasonal calving systems when large numbers of cows are examined in the pre-breeding period, often in a single visit. The requirement of processing and interpreting the slide inhibits real-time cow side decision making about treatment or deciding whether to breed the cow in the upcoming season. The procedure is also much more invasive and must be performed by a well-trained individual in a very hygienic manner and as a result will be more expensive. If subclinical endometritis diagnosis does have a place beyond research, it is likely that it is in targeted individual cows in year-round systems where pre-breeding visits are more dispersed over time, examinations can be repeated and a consistent amount of time exists between parturition and insemination. It is likely for these reasons that rapid cow side diagnostic techniques such as the Metricheck® device and/or transrectal ultrasound to assess uterine content will remain the mainstay of on farm diagnosis for reproductive tract disease beyond three weeks postpartum in seasonal calving systems unless further research on in milk biomarkers becomes more accurate and economically feasible. Consequently, additional alternative research into the existing diagnostic methods is warranted and should be conducted in an Irish context

1.3 Measurement of reproductive performance in dairy cows

Prior to discussion of factors impacting reproductive performance, it is important to review some of the ways in which reproductive performance is measured in the dairy cow. The relevance of these measures to seasonal calving systems as well as the limitations of some of the current measures that are used will also be reviewed.

1.3.1 Measurement of reproductive performance and data required in its determination

Although there are many measures for quantifying reproductive performance, most focus on pregnancy status of the cow (e.g., 'pregnancy rate/risk' or 'calving to conception' are based upon this) once a cow becomes eligible for breeding (Fetrow et al. 1990; Hudson et al., 2012). These aim to determine overall reproductive performance and some have dubbed these primary measures of reproductive performance (Eddy, 1980). Additionally, other measurements of reproductive performance attempt to quantify the reproductive performance based on the two key events that must happen for a cow to become pregnant. The two events that must occur for pregnancy are 1) the cow must express estrus and it must be detected by the farmer or the bull (e.g., 'submission', 'heat detection' or 'calving to first insemination' are based upon this) and 2) the cow must conceive after either an artificial or bull insemination resulting from this estrus event (e.g., 'conception rate' or 'pregnancy per service/AI' are based upon this) (Eddy, 1980; Hudson et al., 2012). Measurements that assess these events are therefore often called secondary measures of reproductive performance. Some of the most commonly used measurements and their definitions are summarized in Table 1.4.

In order to reliably calculate many of these measurements, there is a minimum of baseline data that is required to be recorded on most farms and this includes a calving date, a recorded insemination date (AI or bull) and the pregnancy status (i.e., that is positive or negative status for pregnancy) of the cow relative to that insemination (McDougall, 2006). Insemination date data is available on most farms using AI, however, when natural service with a bull is practiced this information is not always readily available due to fact that bull mating cannot be constantly monitored. This is a common situation in the second half of the breeding season (>6 weeks after MSD) in most Irish seasonal herds. Although to circumvent this problem, an assumed insemination date can be assigned to a cow based on the days of gestation determined by transrectal ultrasound pregnancy diagnosis.

In most modern dairy farms, the pregnancy status following a particular insemination is determined by subsequent pregnancy diagnosis. The type of pregnancy diagnosis method and the operator skill determine the time post AI that this diagnosis will occur (Sheldon et al., 2006b). In Irish dairy cows' pregnancy diagnosis is most commonly determined with transrectal ultrasound after at least one month post AI. Much less commonly, it is performed with transrectal palpation or laboratory tests such as, PAGs (Szenci et al., 1998) or continuous in line P4 assessment in milk (Bruinjé et al., 2019). In some instances, pregnancy status is assumed based on whether a cow fails to return to estrus following a

period of days after AI [e.g., in the case of 56 non-return rates (Jansen and Lagerweij, 1987)] or if the cow re-calves at a time that fits within the normal gestation period following an AI [i.e., in the case of re-calving rates (Ferguson, 2000)]. Understandably the latter methods can be impractical and less accurate than pregnancy diagnosis but still have some use in certain instances (e.g., monitoring 56 non-return rates by AI companies) when pregnancy data is not readily available (Jansen and Lagerweij, 1987).

Whatever method of pregnancy determination is performed, if a particular insemination results in pregnancy, then this date of insemination is commonly referred to as the conception date which corresponds with the day of fertilization (Hubbert, 1972). However, as early pregnancy loss in the dairy cow is high (see section 1.2.4) and pregnancy diagnosis cannot practically be determined by transrectal ultrasound until at least one month after an AI, measurements of reproductive performance that are calculated by this means only quantify cows where fertilization occurs and pregnancy establishes to the point of diagnosis rather than assessing whether fertilization occurred and an early embryo developed but was lost before diagnosis (Morton, 2004). This is also likely part of the reason that there has been ongoing confusion in describing the measurements that quantify the likelihood of pregnancy occurring after an insemination as either conception rate or pregnancy rate (Hudson et al., 2018). More recently, the term, pregnancy per serve (AI) has been used to minimize this confusion in terms (see Table 1.4 for further description).

Measurements of reproductive performance that are calculated from this above information can be generally categorized as either interval based or rate (or 'risk') based and important measurements are summarized in Table 1.4.

Table 1.4. Summary of a selection of commonly used measurements of reproductive performance used in dairy cows. A detailed explanation and the limitations for each with associated references is provided in the text. **what constitutes cows as becoming eligible is discussed in the text of the thesis (see section1.3.3)*

Measurements	**Definitions**
Interval Based	
Calving Interval	Days between two successive calving dates.
Calving-to-conception interval	Days between calving date and date of conception.
Days Open	*For pregnant cows*; Days between calving date and date of conception (*Year-round*); Days between MSD and date of conception (*Seasonal*) *For non-pregnant cows*; Days between calving date and date of when became ineligible for breeding (*Year-round*); Days between MSD and MED (*Seasonal*).
Calving-to-first service interval	Days between calving date and date of first insemination.
Rate or risk based	
Pregnancy per serve (AI) **Or "*Conception/Pregnancy Rate*"**	Proportion of total pregnancies over the total number of inseminations
First Service Submission rate **Or "*Insemination risk*"**	Proportion of cows that are first inseminated over all the cows that were eligible* for their first insemination in a defined period (21 or 24 days). For year round herds this is the 21/24 day period after the end of the voluntary waiting period. For seasonal herds this is the 21/24 day period after MSD.
21 day pregnancy risk	Proportion of cows that become pregnant over all the cows that were eligible* to become pregnant in a defined 21 day period.
100-day in calf rate	Proportion of cows that become pregnant over all the cows that were eligible* to become pregnant by the first 100 days in milk.
6-week in calf rate	Proportion of cows that become pregnant over all the cows that were eligible* to become pregnant within 6 weeks after MSD.

1.3.2 Interval based measurements of reproductive performance

Interval based measurements of reproductive performance describe the time interval between two events (e.g., calving interval is defined as the time between two successive calving 'events' or dates) (Eddy, 1980; Fetrow et al. 1990; Cook, 2010). The most common interval-based measurements are calving interval, calving to conception calving to first insemination/serve and days open (Fetrow et al. 1990; Meadows 2005; Cook 2010). The calving-interval calculation's reliance on subsequent calving, means there is a large time lag before it can be calculated and when it possible to calculate, it is a reflection of past rather than current fertility and its use is limited at best (Cook, 2010). Calving-to-conception suffers from less time lag effects and is a more useful current measure of reproductive performance (Cook, 2010). It assesses both the efficiency of estrus detection and the ability of the cows to establish pregnancy following AI by quantifying the time to conception in each cow. Calving to first insemination is used to assess the efficiency of the estrus detection on the farm by quantifying the time taken by each cow to be inseminated on farm and is a reasonable assessment of heat detection efficiency and resumption of cyclicity in a farm (Cook, 2010).

With interval-based calculations, often the mean value of the herd or a particular cohort is calculated and compared to a specific target for that system [e.g., a calving interval of 365 days is deemed ideal for a cow in a seasonal system (McDougall, 2006)] to examine if the mean herd performance is meeting the pre-defined target. As interval data is often not normally distributed and tends to have a positive or right skewed distribution, assessment of the mean does not always reflect the central tendency of a cohort and the median is often more appropriate to assess (Hudson et al., 2012).

Another major limitation of many of these interval measures, however, is the fact that an event must occur for the measurement to be calculated. For example, for the calving-to-conception interval, a cow must be positive for pregnancy and thus have an assigned date of conception to calculate it. Therefore, a cow that is negative for pregnancy and thus has no assigned date of conception will not be included in this calving-to-conception interval calculation (Eddy, 1980; Cook 2010). The same is true for other common interval-based measurements such as calving to first serve (i.e., it excludes those cows not yet inseminated but eligible to be inseminated) and calving interval (i.e., excludes those that do not re-calve despite being eligible to be bred, conceive and re-calve) (Olori et al., 2002). Consequently, interval-based measurements can be inherently biased as they may not account for cows eligible for an event unless an event has occurred.

Some other interval-based measurements, such as days open have accounted for cows that were eligible but did not conceive and as a result were previously excluded from metrics like calving-to-conception interval (Fetrow et al. 1990; Meadows 2005). Days open is calculated as the number of days from calving to conception for those cows conceiving and from calving to when they no longer are deemed eligible for breeding for those cows that did not conceive (Fetrow 1993; Meadows 2005). A modified version

of this metric for seasonal systems, has been also developed based on number of days from MSD to conception for those cows conceiving and from MSD to MED (i.e., the time period they were eligible to be bred) for those cows who did not conceive (Grosshans et al., 1997) but its' use is not widespread.

Most of the interval-based metrics of reproductive performance are used more commonly in year-round calving systems as opposed to seasonally calving systems. One of the main reasons underlying this is that in year-round calving systems, the time or interval between calving and breeding (AI) or conception is based on each individual cow and is generally a fixed time range (Fetrow, 1993). In year round calving systems, cows will be often become eligible to be inseminated after a fixed period of days after calving which typically range from 45-60 DIM but the exact time is very much farm dependent (Fetrow, 1993). This fixed period where the farmer decides not to breed is called the voluntary waiting period (**VWP**) and once elapsed, the cow becomes eligible to be inseminated and conceive (Fetrow et al., 1990). Therefore, interval-based calculations suit year-round calving systems as if the VWP is the same fixed DIM for each cow on a farm, calculation of the calving to first service or calving to conception interval is only assessing the time taken from when the cow becomes eligible (i.e., end of the VWP) to the event (i.e., insemination or conception).

These measurements are therefore not as useful in seasonal herds and should be interpreted with caution in mind. In seasonal systems as the period of days from calving to breeding and/or conception is highly variable and largely dependent on the cow's calving date relative to herd MSD, earlier calving cows will have a longer period of time when there is no potential of conceiving (i.e., as no breeding is taking place) versus that of a later calving cow. For example, if breeding commences on May 1st and one cow calves on February 1st and another on April 1st the minimum period of days before she can be possibly bred is 89 DIM and 30 DIM, respectively. Additionally, there is also a strong tendency for these earlier calving cows to conceive in a short period of time after MSD (McDougall, 2001a) as they have had longer to resume cyclicity and clear uterine infections in this long waiting period prior to breeding. Due to these issues comparing cows in seasonal herds by analyzing the mean period of time from calving-to-conception is therefore problematic. A modification of the days open [i.e., MSD to conception for those conceiving cows and MSD to MED for those not conceiving(see Table 1.4)] is therefore a more interpretable time-based metric in seasonal calving systems, although account for date of calving is still likely required.

1.3.3 Rate or risk based measurements of reproductive performance

Many of the rate-based measurements of reproductive performance assessment are more appropriately described as risks (Hudson et al., 2018). A rate is defined as the frequency of an event per unit population per unit time while a risk is the frequency of an event, given a designated number of times when it could happen (Hudson et al., 2018). As many of the traditionally called 'rate' based measurements are

determined as the event (e.g., pregnancy) as the proportion of the times it could occur (e.g., when an insemination is administered), it would therefore be more appropriate to term them risks. However, despite efforts being made to rectify this, the tradition of describing many of these metrics as rates has persisted. Throughout this thesis, definitions and efforts to clarify what is being quantified will be indicated to the reader (see Table 1.4 for definitions of commonly used terms).

There are many rate/risk-based metrics that describe and the likelihood of insemination in a defined period of time, the likelihood of conception (or pregnancy) following an insemination and as an overall measure of reproductive performance, the likelihood of pregnancy in a defined period of time (Fetrow et al. 1990; Cook 2010). Many of these metrics are used in both year-round and seasonally calving systems and have fewer limitations than that of the interval-based metrics. However, there are considerable differences in both terminology, calculation and the targets between these systems.

Those metrics that assess the likelihood of insemination (or 'submission') are calculated as the proportion of cows that are inseminated over all those cows that were eligible to be inseminated in a defined period, typically the length of a estrous cycle (i.e., 21 or 24 day period) (Cook 2010; Hudson et al., 2018). These metrics are a reflection of the estrus detection efficiency (i.e., the number of estrus events that are identified) by the farmer and especially in the case of the first service submission rate may also be influenced by the proportion of cows that have resumed cyclicity. In year-round herds, the first serve submission ('insemination') rate/risk is calculated as the proportion of cows that that are inseminated over all those cows that were eligible to be inseminated in the first 21 or 24 days after their VWP of the farm (Cook, 2010). In seasonal herds, the first insemination submission rate/risk as the proportion of cows that that are inseminated over all those cows that were eligible to be inseminated within either the first 21 or 24 days after MSD (McDougall, 2006).

Those metrics that assess the likelihood of conception (or pregnancy) following an insemination are calculated as the proportion of cows that are diagnosed pregnant over the inseminations that occurred (Fetrow et al. 1990; Cook 2010). This has been commonly referred to as 'conception rate' or 'pregnancy rate' but is more appropriately described as pregnancy per serve (or AI) (Hudson et al., 2018). The same calculation is used in both year and seasonal systems and is an informative metric as it indicates the probability of pregnancy following an insemination. It can be calculated as an overall pregnancy per AI figure, or it can be assessed in a cohort of cows, over a time period, related to an AI effect (e.g., based on an AI technician or bull code) or by any other available variables (Fetrow, 1993). The first service pregnancy per AI avoids much of the potential for confounding due to different management policies in different farms and in seasonal herds is often the most useful and reliable (Morton, 2004), as after the second service, remaining cows will be bred by bulls and these services are often not recorded.

Many of the rate/risk-based metrics that assess pregnancy status performance aim to describe overall reproductive performance as opposed to deciphering whether the performance is due to either a

decreased likelihood of insemination or conception. These metrics also differ markedly between year-round and seasonal systems. The 21-day pregnancy risk, 100-day in calf rate and 6-week in calf rate are some of these commonly used metrics and the definitions are outlined in Table 1.4. In year-round systems, the 21-day pregnancy risk is expressed as a proportion or percentage of cows that become pregnant over all the cows that were eligible to become pregnant in a defined 21-day period (Cook, 2010). It describes the probability or risk that a cow who is past her VWP and eligible to be bred, will be bred and establish a pregnancy in the defined time frame. It is a useful overall metric for year-round systems as it is easy to calculate on an ongoing basis and only requires determination of eligibility for breeding and sound pregnancy status data. Likewise, the 100-day in calf rate which describes the proportion of cows eligible for breeding that conceive within the first 100 DIM is also easy to calculate and only requires eligibility and pregnancy status data. In seasonal systems, neither of these metrics are routinely calculated owing to the fact the 6-week in calf rate is easier to calculate, it provides a good reflection of next year's calving pattern and is unaffected by variable breeding season length (i.e., all breeding seasons will be greater than 6) (McDougall, 2006; Macmillan, 2012). The 6-week in-calf rate is calculated as the proportion of cows eligible for breeding that become pregnant in the first 6 weeks after MSD and the proposed target is ≥75% of the herd in-calf in 6 weeks after MSD for seasonal herds (Macmillan 2012).

One of the major issues with many of the rate/risk-based metrics in both year-round and seasonal systems, is that there is a requirement to identify cows that are eligible for breeding (Cook, 2010). Evidently, variation between criteria to determine eligibility may alter the denominator value and ultimately the proportion calculated, making comparisons between herds and research challenging. In the context of both year-round and seasonal herds, farmers should select cows prospectively for breeding, i.e., they should select those intended for breeding and mark those not intended for breeding (so called voluntary culls) before either the end of the VWP or the MSD, respectively (Lindley and Willshire, 2020). However, retrospective removal of cows or the fact that some cows will become ineligible for breeding during the defined breeding period but are not identified as such have the potential to skew the resultant reproductive measurement (Lindley and Willshire, 2020). Additionally in seasonal herds, another important consideration that surrounds eligibility is that of late calving cows in the early part of the breeding season. As MSD is what defines the commencement of breeding in a seasonal herd, it will be possible that many late calving cows have the potential to be bred from very early post-partum e.g., a cow calving on 15th of April when May 1st is the herd MSD would mean that she is 16 DIM when breeding commences. The issue that arises with these cows is determining at what DIM is it appropriate for them to be become eligible for breeding, and as VWP are not often used in seasonal herds and the days after calving when a farmer is willing to inseminate a cow likely varies herd to herd and possibly even breeding season to breeding season. As the probability of submission for insemination and conception is reduced in later calving cows (see section 1.4.2.1) and many of the calculated metrics

do not always account for theses cows, it is an important point to remember when assessing this reproductive performance measurement in seasonal calving herds and cows.

1.3.4 Measurement of estrus detection accuracy

As previously stated, reproductive performance measurements such as submission (insemination) rate/risk indicate the likelihood a cow will be submitted for an insemination in a defined time period (Cook, 2010). This metric is therefore reflective of the estrus detection efficiency on farms. However, this metric does not inform as to whether that a cow's insemination was performed following a true estrus event (Hudson et al., 2012; Smith et al., 2014). In the case of herds where estrus detection is not accurate, the submission rate may be high, but a high number of inseminations are likely being performed when cows are not truly in estrus (Macmillan and Watson, 1976; Hudson et al., 2012). One practical on farm approach to measuring the accuracy of estrus detection is via the retrospective calculation of the intervals between successive inseminations (Hudson et al., 2012; Smith et al., 2014).

As typical estrous cycles last between 18-24 days (see section 1.2.1), most cows that fail to conceive should return to estrus in this day range after AI. Cows that are missed by the farmer at a subsequent estrus may be inseminated again at second subsequent estrus i.e., double this normal interval or 36 to 48 days. All these normal inseminations intervals indicate correct identification of an estrus event, although an increased proportion in the 36 to 48 days range can indicate that a higher number of estrus events are being missed (Smith et al., 2014).

However, if the interval between two successive inseminations does not fit within these expected normal estrus ranges (i.e., short abnormal repeat range of 2-17 days or a long abnormal repeat range of 25-35 days) it can indicate that an insemination and hence an estrus event was detected inaccurately (Smith et al., 2014). When all repeat intervals are assessed and there is found to be an elevated proportion in these abnormal categories it can indicate a management issue with estrus detection accuracy(Smith et al., 2014).

Insemination interval analysis can also indicate that there may be an issue with late embryonic or early fetal death if there is a high proportion of intervals in all categories greater than 24 days (Smith et al., 2014). Additionally, recent work might suggest that in certain populations of cows (e.g., UK dairy cows) that the normal interservice interval may extend past 24 days to 25 or 26 days (Remnant et al. 2015). The interpretation of insemination analysis is indicated in Figure 1.4.

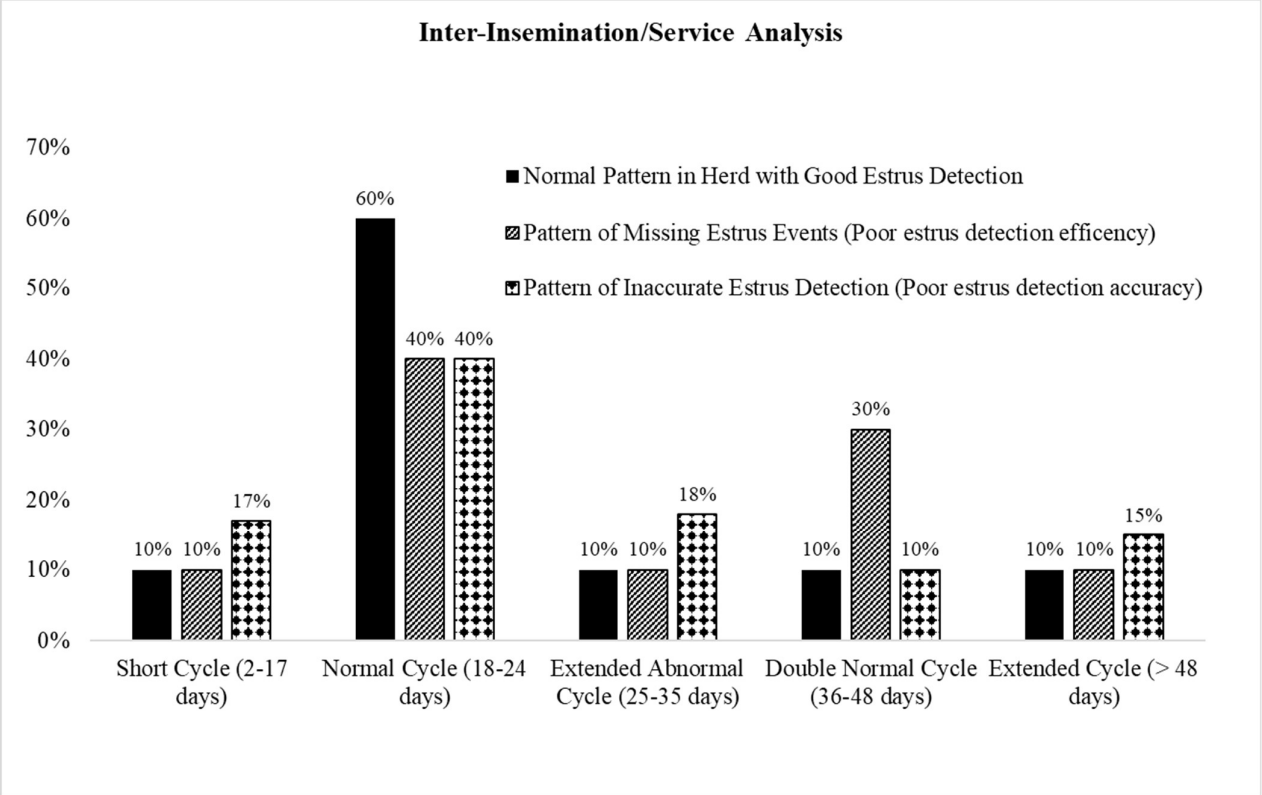

Figure 1.4 The interpretation of inter-insemination/service analysis. Ideally, most (>60%) repeat intervals should occur at a normal (18-24 days) period post insemination for non-pregnant cows. Double Normal (36-48 days) intervals indicates that an estrus was missed and high proportion can indicate a herd problem with estrus detection efficiency. Short (2-17 days) or Long Abnormal (25-35 days) intervals indicate that an estrus event was incorrectly identified and a and high proportion can indicate a herd problem with estrus detection accuracy. Late embryonic or early fetal death can cause high proportion of intervals in all categories > 24 days. Adapted from Hudson et al., (2012) and Smith et al., (2014)

1.3.5 Summary of the measurements of reproductive performance suitable to cows in seasonal calving systems.

As outlined in the previous sections, interval measures or those measures based on calving date are not appropriate to seasonal calving systems due to their limitations relating to the temporal influence of calving date relative to MSD, their exclusion of certain cohorts of cows and the lag effects of certain measurements. In terms of primary measures of reproductive performance, the most important in seasonal calving herds are the 6 week in-calf rate (i.e., the proportion of cows that become pregnant over all the cows that were eligible to become pregnant within 6 weeks after MSD and the target is >75%) and the overall proportion of cows in-calf by the end of the breeding period, although the latter is less useful as it is influenced by the duration of the breeding period. These were two of the two primary measurements that were found by the In Calf Project developed in Australia (Morton, 2000) and are widely used in seasonal calving systems today. The same project also recognized that these primary measurements were influenced by the secondary measurements of 21 or 24-day submission rate from MSD (i.e., the proportion of cows that were inseminated in the first 21 or 24 days of the breeding season and the target is >90%) and the overall pregnancy per AI or 'conception rate' (i.e., the proportion of pregnancies over the total number of first AIs and the target is ~60%). Thus the key factors that influence reproductive performance in seasonal calving systems are those that impact on either submission or conception rates or both and thus warrant further mention.

1.4 Factors impacting reproductive performance in seasonally calving pasture-based dairy cows

As the establishment of pregnancy is a complex and interlinked phenomenon, it is unsurprising that a multitude of factors impact on reproductive performance in individual dairy cows. This section will aim to outline some of the factors that impact reproductive performance in seasonally calving pasture-based dairy cows with reference to Irish systems where possible.

1.4.1 Herd versus cow level factors and reproductive performance

Firstly, it is important at this point to differentiate between herd and cow level factors that influence reproductive performance at both herd and cow-level, respectively. Cow-level factors or variables are those that are specific to individual cows or even an individual lactation within cows and impact on an individual cow's subsequent reproductive performance. Common identified cow or lactational-level factors impacting reproductive performance in seasonal calving systems includes BCS loss in early lactation (Buckley et al. 2003; Roche et al. 2007), calving date relative to MSD (McDougall, 2001a; Lane et al., 2013), milk composition particularly protein concentrations (Morton et al., 2016), parity/age (Carty et al., 2020) and reproductive tract diseases (McDougall et al., 2011).

Herd-level factors or variables are those that are specific to a herd or farm and impact overall herd reproductive performance. These herd-level factors will often be environmental and management practices that are directly [e.g., the 'sensitivity' or efficiency of the herd's estrus-detection systems (Morton, 2010)] or indirectly [e.g., herd nutritional management (Butler, 2014)] related to reproduction. Common identified herd-level factors in seasonal calving systems include existing calving pattern on farm, estrus detection efficiency (i.e., efficiency of management methods used to detect estrus), AI and bull breeding management, negative energy balance in the herd in early lactation and inadequate replacement heifer rearing strategy (Macmillan 2012).

Many of the studies of reproductive performance, are conducted at the cow or lactational-level with much fewer studies conducted at herd-level (Morton, 2004). Some cow or lactational-level factors that impact on reproductive performance will only have an effect at herd-level if a high proportion of cows suffer the same problem or exposure (Macmillan, 2012). An example may relate to marked BCS loss in early lactation which occurs at an individual, lactational-level and will impact on subsequent reproductive performance at the cow level (Buckley et al., 2003) yet if there is a herd issue [e.g., with lack of nutritional energy supplied in early lactation, (Butler, 2014)] that results in marked BCS loss in a high proportion of cows, then this will also impact reproductive performance at the herd level also. However, herd-level factors that impact herd level reproductive performance may not always be the same as those noted at the cow or lactational-level and vice versa. Both of these problems of incorrect inferences based on the observed data are referred to as either 'atomistic' and 'ecological' fallacy (Diez Roux, 2002). Atomistic fallacy refers to when an incorrect inference is made about a relationship between groups or herds based on analysis of individual or cow-level data while ecological fallacy is

when incorrect inferences are made between individuals based on analysis of group or herd-level data (Diez Roux, 2002).

For farmers, herd reproductive performance is often the most important monitored aspect as it relates to profitability but as researchers, the majority of studies on reproductive performance are based on cow or lactational-level studies. Therefore the issue of atomistic fallacy is a more likely pitfall if these results are used to advise farmers on methods to improve herd reproductive performance (Morton, 2004). That said, further cow-level research based on some of the known factors which impact individual reproductive performance is still warranted as it contributes to the knowledge base of advisors in terms of offering guidance to farmers in areas related to reproductive performance in individual cows.

The research presented in this current thesis relates two elements related to reproductive performance; reproductive tract disease and estrus detection inaccuracy. Both elements are investigated at the individual cow-level and not herd-level and thus the inferences made should be interpreted as such. Additionally, further review of the literature will only focus on the key cow-level factors impacting reproductive performance.

1.4.2 Cow/lactational-level factors impacting reproductive performance

Many of the key factors impacting reproductive performance at the cow-or lactational-level have been noted in the previous section (section 1.4.1) and the physiological basis behind their effects is noted throughout section 1.2. The following section will serve to summarize the research and explain the interrelationships of some of the most important of these in the context of seasonal dairy cows.

1.4.2.1 Calving date relative to MSD

Reproductive performance of cows far from the MSD is superior to that of those calving close to or after the MSD. This is a well-recognized problem in seasonal calving herds and there has been extensive research to illustrating the impact this has on reproductive performance in New Zealand (McDougall, 2001a), Australia (Morton, 2011) and Ireland (Lane et al., 2013, Carty et al., 2020). These cows will has less time to resume cyclicity, restore the endometrium and eliminate bacterial contamination of the reproductive tract before breeding commences (see section 1.2). Additionally, there is a potential risk that later calving cows will be at increased risk of higher BCS due to a prolonged dry period which in turn may predispose to an increased risk of metabolic diseases (Roche et al., 2013a) and impaired reproductive performance. For this reason, many have advocated the use of ovulation synchronization protocols in late calving to hasten the resumption of cycility and in order to ensure they are inseminated within the first 6 weeks of the breeding season (McDougall 2010; Herlihy et al., 2011).

1.4.2.2. Metabolic diseases

BCS and bodyweight losses can indicate an increase in the severity and duration of the post-partum negative energy balance (Roche et al., 2007). In both New Zealand (Roche et al., 2007) and Ireland (Buckley et al., 2003), research in seasonal calving dairy cows has described the relationship between postpartum BCS losses and reproductive performance. In both studies, all reproductive measures were negatively associated with BCS loss between calving and breeding. Consequently, there is strong evidence to advise that seasonal calving dairy cows should minimize post-partum BCS loss to less than or equal to 0.5 BCS units in Ireland (Buckley et al. , 2007) and no more than 1.5 units in New Zealand (Roche et al., 2007). The physiological factors behind NEB and reproduction performance have been already alluded to (see section 1.2) but it is important to stress that as well as effects of delaying the resumption of cyclicity and lasting negative impacts on the oocyte, NEB post-partum also predisposes to immunosuppression and predisposes to reproductive tract disease development. This explains why it is also an established risk factor for reproductive tract diseases in studies of seasonal calving dairy cows (Giuliodori et al., 2017).

The association with hypocalcaemia and reproductive outcome in seasonal dairy calving cows is not as well described. Recently, researchers (Umaña et al., 2018) found that subclinical hypocalcaemia diagnosed on the day of calving was associated with reduced fertility in a herd of grazing dairy cows. In the same study, no association was found with subclinical hypocalcaemia and clinical endometritis but an association been noted in other studies (Whiteford and Sheldon, 2005) in year-round herds. Further observational research on clinical and subclinical hypocalcaemia in grazing cows is necessary to better understand these interrelationships but is particularly challenging in an Irish context due lack of farmer recording of clinical cases and logistical issues around the tight sampling window as cows would require calcium status determined within 24 hours of calving and this could be logistically challenging for a small research team.

1.4.2.3 Parity

Reproductive performance of older, multiparous cows is inferior to that of younger, primiparous cows. Depending on the research, effects appear to become most evident from 4th or 5th parity onwards (McDougall 2001a; Morton 2011; Carty et al., 2020). Less commonly replacement heifers of inadequate bodyweight can have reduced reproductive performance (Macmillan, 2012) and are worthy of a mention. In seasonal calving herds older multiparous cows are often later calving and thus their reproductive performance is confounded by the shorter interval from calving to MSD (McDougall, 2001a). Likewise, increasing parity also increases the likelihood of pregnancy loss (López-Gatius et al., 2002), twinning (Fricke, 2001), retained fetal membranes (Laven and Peters, 1996), cystic ovarian disease (Vanholder et al., 2006), reproductive tract diseases [i.e., metritis (Bruun et al., 2002), PVD

(Potter et al., 2010)] and lameness (Somers et al., 2019). All of these conditions will in their own right impact on reproductive performance and are worth keeping in mind.

1.4.2.4 Calving related factors and reproductive tract diseases

Many calving related factors such as abortion, induced parturition, dystocia (or assisted calving) and retained fetal membranes have been negatively associated with reproductive performance in seasonal calving dairy cows (McDougall, 2001a). In the same research (McDougall, 2001a), twinning was not found to have an impact on reproductive outcome in seasonal calving cows, but other researchers have described a negative association in year-round herds (Fricke, 2001). Additionally, it is known that twinning and many of the above listed factors are also risk factors for reproductive tract disease development (see Table 1.2 also). There is a wealth of evidence to indicate reproductive tract diseases, such as PVD and subclinical endometritis (McDougall et al., 2007, McDougall et al., 2011) (see section 1.2.8) impact reproductive performance in seasonal dairy cows, mediated through a reduction in both the probability of submission for insemination and conception post-insemination.

Clearly, there are numerous interrelationships between transitional cow or calving related factors and reproductive tract diseases with some factors predisposing to another. Some of these factors may be associated directly with impaired reproductive performance or they may be indirectly associated through predisposition to another important factor impacting reproduction. As many of these factors are interlinked and it can pose a challenge to analyse the true effect of a variable with many confounding factors. One of the methods to help decipher relationships between variables is casual pathway analysis and the construction of directed acyclic graphs (Shrier and Platt, 2008). This approach has been used to model the interrelationships between various factors and reproductive performance in seasonal calving cows before (McDougall, 2001a). Casual path analysis and directed acyclic graphs assist in model construction, are based on previous knowledge of an interrelationships between factors and should have a sound biological basis (Shrier and Platt 2008). Using this approach to confounding and selection bias is therefore more likely to reduce the degree of bias for the effect estimate in the chosen hypothesized model over the more traditional methods, such as multivariable analysis, of model construction (Shrier and Platt 2008). Casual analysis is therefore a logical approach to take when analysing reproductive outcome in seasonal dairy cows.

1.4.2.5 Anestrus and cystic ovarian disease

Dairy cows that have an extended time to first ovulation are less likely to establish pregnancy early in the breeding season. In pasture-based cows the period to first ovulation has been estimated to occur with a range of 24-43 DIM (McDougall, 1994; O'Sullivan et al., 2020; Meier et al., 2021) while in a review of anestrus, Rhodes et al. (2003), noted that anywhere between 13-48% of dairy cows had not had an observed estrus by MSD. Reproductive performance is reduced in those seasonal calving cows treated for anestrus (McDougall, 2001a). Likewise, cows diagnosed with cystic ovarian disease have been

shown to have reduced reproductive performance (Cattaneo al., 2014). As discussed as part of the physiological review (section 1.2.6), the complex interrelationship between uterine inflammation and ovarian events means that estrous activity may be inhibited by reproductive tract disease and this interrelationship also should not be neglected.

1.4.2.6 Lameness

Lameness is well-recognized to impact on the fertility of dairy cow through delaying the resumption of cycility (Garbarino et al., 2004) and through a reduction in estrus behaviour (Walker et al., 2008) when these cows do resume cyclicity. This results in a missed estrus events and an increase in treatments for anestrus by farmers (Hultgren et al., 2004). Studies from both Ireland (Somers et al., 2015) and New Zealand (Alawneh et al., 2011) have illustrated the association between lameness and reproductive performance in seasonal calving pasture based dairy cows. Timing of the lameness event is important with those cows becoming lame after the earliest serve date and those cows that were lame both before and after the start of breeding, being the cows that were impacted (Somers et al.,, 2015). Like many other factors already discussed, there is also an interrelationship between excessive BCS or bodyweight loss in early lactation and lameness risk in seasonal grazing dairy cows (Alawneh et al., 2014; Somers et al., 2019) owing somewhat to loss of the fat in the digital cushion (Green et al., 2014).

1.4.2.7 Individual Genetic Values for Fertility

Multi-trait selection indexes have been widely adopted in modern dairy production systems (Cole and VanRaden, 2018). In the Irish context, this system is called the Economic Breeding Index (**EBI**) and allows for selection for multiple traits such as milk production, fertility, calving performance, beef carcass, cow maintenance, cow management and health (see Figure 1.2) (Berry et al., 2007). Both milk production and fertility are the most heavily weighted traits at 33% (Berry et al., 2007). The fertility subindex is based on economic weighting given to the predicted transmitting ability of two traits; calving interval and survival (Berry et al., 2007). Calving interval is a measure in difference in days between successive calving versus a baseline cow and survival is a binary trait based on the on a cow surviving from lactation to lactation (Berry et al., 2007). Calving interval is used as it is the easiest available reproductive data that is available continuously in a large population data set and survival is included to account for cows that did not re-calve and therefore were not included in the genetic evaluation for calving interval (Berry et al., 2007). Despite the fact that heritabilities for various fertility traits are relatively low [0.01 to 0.07, (Veerkamp et al., 2001)], numerous studies in Ireland have illustrated that the fertility subindex was positively associated with hazard of pregnancy within a breeding season (Carty et al., 2020; O'Sullivan et al., 2020). Additionally, other Irish studies illustrated that cows with superior genetic merit for these traditional reproductive traits (i.e., calving interval) have been associated with improved reproductive phenotypes such as the resumption of cyclicity and uterine scores (Carthy et al., 2016).

1.4.2.8 Milk yield and milk composition

The impact that the individual cow's milk yield has on reproductive performance has been studied over many years and the relationship is somewhat equivocal. Traditionally, increased milk yield has been associated negatively with reproduction (Darwash et al. 1999; Royal et al., 2000). As the genetic correlation between milk yield and fertility is negative (Pryce and Veerkamp, 2001), the selection in the past for milk yield through Holstein genetics was associated with this general decline in fertility. Other issues that might explain this association was that many previous studies have investigated associations between lactational milk yields and reproductive performance, whereas milk yield estimates in early lactation are less likely to bias the association as milk yield is reduced by effects of pregnancy (Roche, 2003). Indeed, when early lactational yield was investigated in the Irish context, there has been a positive association with milk yield and reproductive performance (Buckley et al. 2003; Carty et al., 2020). Buckley et al. (2003) found that when the proportion of Holstein-Friesian genes were adjusted for, there was positive association between milk yield variables at first insemination and reproductive performance while Carty et al. (2020) found a 1 to 1.4 percentage unit increase in the hazard of pregnancy for every 1-kg increase in yield at test day between 0 and 90 DIM. This finding could be explained by the fact that NEB was better managed in cows in these herds might be reflected by increased milk yield and fertility performance (Carty et al., 2020).

The impact of milk constituents such as milk protein, fat, lactose and somatic cell count have all been investigated by various researchers to assess their association with reproductive outcome. Depression in early lactation milk protein levels can indicate NEB (Friggens et al. 2007) while increases in milk fat can indicate excessive fat mobilization (Bauman and Griinari, 2003) and a change in the ratio between the percentages of both that is greater than 1.4 also can indicates NEB (Heuer et al., 2000). Milk protein concentration has been positively associated with reproductive performance in seasonal calving dairy cows in many studies (Buckley et al., 2003; Morton et al., 2016; Carty et al., 2020). In the latter study (Carty et al., 2020), the association between milk fat concentration and reproductive performance was also present but showed a limited increase (2-3 days) in median time to conception. However, in the same study (Carty et al., 2020), milk lactose concentration assessed at between 0-30 DIM had the greatest effect in determining fertility outcome out of all of the milk constituents assessed. As lactose is synthesized from blood glucose (Reksen et al., 2002), early lactational milk lactose concentration is likely associated with reproductive performance as it may serve as a proxy for NEB. Elevated SCC has been associated with reduced reproductive performance seasonal dairy cows too (Carty et al., 2020). Work in the UK (Hudson et al., 2018), has shown both clinical and subclinical mastitis in the time period before and after breeding is negatively associated with pregnancy outcome. The exact mechanism behind this is uncertain but likely relates to the effects of inflammation on oocytes, early embryo development (Soto et al., 2003) and on luteotropic support of the CL (Neuvians et al., 2004). Although many associations between milk composition have been recently described in large population of Irish

dairy cows, the authors concluded that their relative impact was low when they were compared to the impact of genetic values for fertility were also considered (Carty et al., 2020).

1.4.2.9 Inaccurate insemination following an inaccurate or false estrus event

Inaccurate [or 'false positive (Morton, 2004)] inseminations (AIs) refer to when an AI is performed on a cow following detection of a false estrus event. (i.e., ovulation is not imminent) (Roelofs et al., 2010). In these cases, dairy cows who are inseminated inaccurately are either in the luteal phase of their estrous cycle or already pregnant. Indeed, it has been reported that approximately 6% of pregnant cows will illustrate estrus behaviours (Erb and Morrison, 1958; Williamson et al., 1972) and could be inappropriately inseminated. Although some authors (Morton, 2004), have considered inaccurate insemination as insemination-level factor rather than a cow-level factor, it is most likely that inaccurate inseminations impact reproductive performance mainly at the cow-level rather than herd-level, unless there is a large proportion of inseminations conducted inaccurately in a herd due to poor estrus detection methods (see section 1.2.2 and 1.3.4).

The impact that inaccurate inseminations have on cow-level reproductive performance is mainly due to the disruption of an existing pregnancy in a previously inseminated cow as opposed to a luteal insemination in cow that has not been previous inseminated (Macmillan et al., 1977). The proportion of pregnancy losses due to inaccurate inseminations vary from 13 to 29% (Macmillan et al., 1977; Weaver et al., 1989). The degree of pregnancy losses is also dependent on the days when the inaccurate insemination occurs relative to the stage of gestation (Macmillan et al., 1977) and the site of semen deposition in the reproductive tract [i.e., intrauterine versus mid-cervical (Weaver et al., 1989)]. If the inaccurate insemination is conducted 3 and 24 days after an accurate insemination and the cow has conceived, the probability of disruption of an establishing pregnancy is increased (Macmillan et al. 1977; Weaver et al., 1989). Additionally, this probability is further increased if the inaccurate insemination is performed at the uterine body versus the mid cervix (Vandemark et al. 1952; Weaver et al., 1989). Despite, the reduced probability of pregnancy associated with intracervical insemination versus intrauterine insemination in the body (Weeth and Herman, 1951), most AI technician are trained to perform intracervical insemination in cows on repeated inseminations especially if they are unsure of the estrus status (Select Sire Inc., 2020). In terms of seasonal calving cows that typically only have a short period of AI (approx. 6 weeks), the potential to disrupt pregnancies would most likely be at second insemination following the first AI and a disruption at this stage could hamper a cow's ability to re-conceive in the short period of time afterwards as the herds' breeding season is so condensed (McDougall, 2006).

The proportion of inaccurate inseminations in Irish dairy cows are largely unknown and the association with reproduction performance untested. Older estimates, mainly based in Northern Ireland, suggest that somewhere between 7.7-19.7% of all insemination were inaccurate (McCaughey and Cooper, 1980;

White and Sheldon, 2001). Studies that have estimated the prevalence of inaccurate inseminations typically measure P4 in milk or blood at the time of AI (Sturman et al., 2000, Ingenhoff et al., 2016). In a cow that is truly in estrus, P4 should be low (see section 1.2) while those that are in the luteal phase or pregnant should have high P4 (Ingenhoff et al., 2016). However, this test has limitations in terms of accuracy as some cows will have low circulating P4 due to being anestrus or in the period close to estrus (i.e., periestrus) but ovulation will be imminent while others may have elevated P4 and still conceive (White and Sheldon, 2001). Consequently, many of these studies reporting on the prevalence of inaccurate estrus detection and resulting inaccurate inseminations are based on apparent prevalence as they have not adjusted for the test parameters (i.e., sensitivity and specificity).

Inaccurate inseminations mainly occur as a result of poor recognition estrous signs by the farmer due to poor training or inadequate time spent in visual observation (Roelofs et al., 2010). However, inaccurate inseminations may also occur if a cow is genuinely showing some signs of estrus despite being in the luteal phase or pregnant or indeed the cow may have a pathology such as cystic ovarian disease that E2 secreting (see section 1.2.6). Lastly, genuine misidentification of a cow by the farmer due to issues with freeze brands or ID marking could result in an inaccurate insemination. Very few studies have examined that if these factors or signs are present or absent on the cows and whether they increase or decrease the likelihood of inaccurate estrus detection and insemination taking place (Reimers et al. 1985; White and Sheldon, 2001). Further research in this area may assist farmers in identification of these cows so cow-side test can be performed or strategies (e.g., performing cervical AI) to limit the effect of an inaccurate insemination could be avoided.

In addition to the cost of pregnancy loss in Irish dairy cows, inaccurate insemination is wasteful in terms of semen. Between January and June of 2019 a total of 726,225 AIs were conducted on dairy cows in the Republic of Ireland (ICBF, 2020b) and if the previous estimates are to be believe this means that somewhere in the region of 55,919 to 143,066 AIs were performed inappropriately annually. If an AI straw alone costs on average €16, this is an economic loss of potentially €894,704 and €2,289,056 annually to the Irish dairy industry on semen alone. This figure of course does not even factor in the cost of AI technicians [average of €15 cost; (IFJ, 2017)] or the waste of time and labor on the farm if DIY AI is performed. Consequently, there is a need to better quantify the proportion of inaccurate estrus detections and inseminations in Irish dairy cows and conduct some initial investigation on factors that might reduce the proportions occurring at cow-level.

1.5 Summary of the research requirements and research objectives

Reproduction in seasonal calving pasture based dairy cows has been well researched over the years ranging from detailed reproductive physiology research to that determining improved measurements of quantifying reproductive performance to identification of a multitude of the key interlinking factors that determine this reproductive performance at the individual cow-level. This literature review has outlined the major areas of this research and outlined where there are particular knowledge gaps and requirements for further research exist particular in terms of reproductive tract disease and estrus detection inaccuracy in the context of Irish dairy cows.

1.5.1 Reproductive tract disease beyond three weeks postpartum research objectives

As outlined in section 1.2.9, there have been challenges relating to definitions and diagnostics relating to reproductive tract disease beyond three weeks postpartum. Two of the most useful and easy to perform cow-side diagnostics include visually assessing vaginal discharge with a Metricheck® device (or PVD scoring) and visual assessment of transrectal ultrasound for the detection of intrauterine fluid that may be indicate of endometritis (UE scoring). Although PVD scoring has quite a bit of research focus, not much of this has been conducted in the context of Irish dairy cows and the research on UE scoring is much less well researched in terms of a diagnostic tests for endometritis. Further useful research in an Irish context on these diagnostic tests would therefore involve determining the risk factors for reproductive tract disease development and establishing the association of various score on reproductive performance as well as determining better estimates of test accuracy (i.e., sensitivity and specificity).

Consequently, using the analysis of a large retrospective observational dataset the research objectives of this part of the thesis were as follows.

- To describe the cow-level apparent prevalence estimates for reproductive tract disease diagnosed by these two diagnostic methods (i.e., PVD and UE scoring) or in combination
- To investigate the risk factors associated with reproductive tract disease if these diagnostic methods were used in isolation or in combination.
- To assess the association between the results of two diagnostic methods at the pre-breeding examination and the hazard of pregnancy within the subsequent breeding season in a cohort of seasonally calving pasture based dairy cows.
- To determine the diagnostic test parameters (i.e., sensitivity and specificity) at the point of sampling of using a Bayesian latent class statistical approach (i.e., a test in the absence of a gold standard)
- To determine the effect of varying positivity thresholds for disease on these diagnostic test parameters.

1.5.2 Inaccurate estrus detection research objectives

Inaccurate estrus detection and the resultant inaccurate artificial insemination has the potential to impact a moderate number of Irish dairy cows annually. As outlined in the literature review, there is a real paucity of research information relating to this aspect of reproduction in Ireland but also internationally with many studies being conducted many years ago. A rational approach to commencing research in this area in Irish dairy cows would first be to robustly quantify the levels that are occurring.

Additionally, it may be beneficial to identify if there are any cow-level factors that would assist farmers in identifying cows that were more likely to not be not truly in estrus at time of AI so they could be checked with a simple cow-side test.

Consequently, using the analysis of two prospective retrospective observational studies the research objectives of this part of the thesis were as follows;

- To estimate the cow-level true prevalence of inaccurate insemination in a population of in Irish milk recording dairy herds using two classes of the observed data (milk progesterone concentration on the day of AI and apparent conception to the sampled insemination) using a using a Bayesian latent class statistical approach.
- To determine potential cow-level risk factors for estrus detection inaccuracy and thus inaccurate insemination in a select population of Irish seasonal calving grazing dairy cows.

Chapter 2

Cow-level risk factors for reproductive tract disease diagnosed by two methods in pasture-grazed dairy cattle in Ireland.

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2.0. Abbreviations used in this chapter

AOR: Adjusted odds ratio

DIM: Days in milk

HF: Holstein-Friesian

RFM: Retained fetal membranes

SD: Standard deviation

PTA: Predicted transmitting ability

RTD: Reproductive Tract Disease

PBE: Pre-breeding examination

PVD: Purulent Vaginal Discharge

UE: Ultrasonographic endometritis

MS: Mucus Score

- This is based on Williams et al. (2005).
- Please see Table 1.3 for a detailed description, although it is also detailed in the text.
- Please note this is synonymous with PVD score as used Chapter 3.
- This has been left as such to keep consistency with published manuscripts.

UTS: Uterine Tract Score

- This was modified from Mee at al. (2009).
- Please see Table 2.1 for a detailed description.
- Please note this is synonymous with UE score as used Chapter 3.
- This has been left as such to keep consistency with published manuscripts.

2.1 Abstract

The cow-side diagnosis of reproductive tract disease (**RTD**) involves identifying the presence of purulent vaginal discharge (**PVD**) and ultrasonographic endometritis (**UE**). The objectives of our study were to obtain prevalence estimates for RTD diagnosed by 2 methods (PVD and UE scoring) and to investigate the risk factors for increased probability of RTD if these methods are used in isolation or in combination. Our retrospective observational cohort study tested the hypothesis that RTD assessed by 2 methods would have similar risk factors, and that those would be mainly cow- and calving-related factors. We analyzed data from 5,049 pre-breeding examinations (**PBE**) from 2,460 spring-calved cows on 8 farms between 2014 and 2018. Cow-related details assessed were days in milk at PBE, breed, lactation number, dry period length, body condition score at calving and PBE, 305-d milk yield, predicted transmitting ability for production and fertility, the presence of a corpus luteum at PBE, and positive diagnosis the previous year. Calving details assessed were type of sire, calf sex, twinning, stillbirth, calving difficulty score, and retained fetal membranes. We conducted statistical analyses using 4 multivariable logistic regression models to identify the risk of RTD diagnosed by (1) PVD in isolation, (2) UE in isolation, (3) the presence of either PVD or UE; and (4) the presence of both PVD and UE. We accounted for herd, cow, and year as random effects in all 4 models. The overall prevalence of RTD in models 1, 2, 3, and 4 were 7.5, 6.7, 11.6, and 2.6%, respectively. Days in milk at PBE, the interaction between days in milk and retained fetal membranes, twinning, and the predicted transmitting ability for calving interval were consistently significant risk factors for positive scores in all 4 models. Considerable calving difficulty [adjusted odds ratio (**AOR**) = 13.64], Holstein Friesian dam breed (AOR = 2.58), first lactation (AOR = 2.39), and body condition score at PBE (AOR = 1.64) were risk factors for a positive PVD score but not for a positive UE score. Fifth lactation (AOR = 1.69), a beef-sired calf (AOR = 1.46), and the absence of a corpus luteum at PBE (AOR = 1.57) were risk factors for a positive UE score but not for a positive PVD score. These results support the hypothesis that most of the risk factors for PVD and UE are the same but some are distinctly different yet all of these findings should be considered for preventative advice and identifying at-risk cows for RTD.

2.2 Introduction

Microbial contamination of the uterine lumen occurs in 80 to 90% of dairy cattle in the first 2 weeks postpartum (Sheldon et al., 2006a). Failure to clear pathogenic bacteria from the uterine lumen after 3 weeks postpartum results in endometritis (Gilbert et al., 2005; Sheldon et al., 2006a). Endometritis causes endometrial tissue damage, and pathogen-associated molecules disrupt endocrine function (Sheldon et al., 2002b; Williams et al., 2007; Herath et al., 2009). Affected cows can experience a delayed resumption of ovarian activity after calving and prolonged luteal phases once they do begin to cycle (Opsomer et al., 2000; Sheldon et al., 2002b). Ultimately, endometritis has been shown in several studies to reduce submission, conception, and pregnancy rates, and to increase culling for failure to conceive (LeBlanc et al., 2002; McDougall et al., 2007, 2011; Šavc et al., 2016).

Clinical endometritis has been defined by the presence of purulent (>50% pus) vaginal discharge 21 d or more postpartum, or mucopurulent (50% pus, 50% mucus) discharge 26 d postpartum without accompanied systemic signs (LeBlanc et al., 2002; Sheldon et al., 2009). Subclinical endometritis is diagnosed based on uterine cytology: more than 18% neutrophils in uterine cytology samples collected 21 to 33 d postpartum, or more than 10% neutrophils at 34 to 47 d (Sheldon et al., 2006a), in the absence of visible pus in the vagina. Cow-side diagnosis of subclinical endometritis with uterine lavage or cytobrush cytology poses substantial practical challenges, including time constraints, laboratory processing, and expense, and it assesses only 1 element of reproductive tract inflammatory disease (Dubuc et al., 2010a; Deguillaume et al., 2012; de Boer et al., 2014). More recently, many authors have moved away from using the term clinical endometritis, preferring the term purulent vaginal discharge (PVD) because it is more descriptive and covers a range of conditions: endometritis, vaginitis, and cervicitis (Dubuc et al., 2010a; de Boer et al., 2014). However, the use of a Metricheck® device (Simcro, Hamilton, New Zealand) in isolation fails to detect cows that have no vaginal discharge but evidence of ultrasonographic endometritis (**UE)** (Šavc et al., 2016). With the widespread use of ultrasonography in dairy reproduction, ultrasonographic scoring systems for assessing endometritis have been studied and shown to be both practical and effective at diagnosing both clinical and subclinical endometritis and predicting reproductive outcomes in cows (Mee et al., 2009; López-Helguera et al., 2012; Meira et al., 2012). Dubuc et al. (2010a) found that cows diagnosed with PVD or subclinical endometritis at 35 DIM had poorer subsequent reproductive performance than unaffected cows, and these effects were additive if both conditions occurred simultaneously. More recently, Šavc et al. (2016) found that a combination of both vaginal mucus scoring and ultrasonography of the uterine tract was better at predicting fertility outcomes than either method used in isolation. Because poorer fertility outcome is predicted by both methods of diagnosis, and because both methods assess different parts of the reproductive tract, we will use the broader term "reproductive tract disease" (**RTD**) to describe both PVD and UE in this report, because it is a more all-encompassing and descriptive term than previous terms such as clinical endometritis.

Globally, the incidence of RTD in dairy cows varies widely, from 5 to 30%. This variation is due to the aforementioned issues around definitions, disease states, timing of examination relative to calving, lack of a gold standard diagnostics, and differences between populations and farming systems (de Boer et al., 2014). Many risk factors for PVD are well established, including DIM at examination, retained fetal membranes (**RFM**), metritis, dystocia, abortion, stillbirth, twinning, male offspring, negative energy balance, and primiparity (Ghanem et al., 2002; Kim and Kang, 2003; McDougall et al., 2007; Dubuc et al., 2010b; Potter et al., 2010; Giuliodori et al., 2013a, 2017). However, of the few studies in which endometritis was diagnosed by ultrasound tract scoring, risk factors identified have been DIM at examination, primiparity, dystocia, and cows with inferior genetic merit for traditional fertility traits (Carthy et al., 2014, 2016; Fitzgerald et al., 2014).

Given that both PVD and UE affect subsequent reproductive performance (Mee et al., 2009; Dubuc et al., 2010a; Šavc et al., 2016) and most studies have focused only on establishing prevalence estimates and risk factors for RTD determined by a single diagnostic method (PVD, UE or subclinical endometritis), the objective of the present study was to establish prevalence estimates and risk factors for the 2 most common cow-side diagnostics, used in isolation or in combination.

In nonseasonal calving herds, pre-breeding examinations (**PBE**) take place before the end of the voluntary waiting period; in seasonal calving herds, PBE take place before the mating start date, or just afterward in a small proportion of later-calving cows (Mee et al., 2009). Investigation of the risk factors for the different diagnostic methods for RTD will improve our understanding of those risk factors, helping to implement better herd-level monitoring and preventative measures, and helping to identify at-risk cows in seasonal calving systems for early intervention and treatment (McDougall et al., 2001b). We hypothesized that RTD as assessed by Metricheck® and ultrasound would have similar risk factors, and that these risk factors would be primarily cow and calving-related factors.

2.3 Materials and Methods

2.3.1 Animals and Herds

This retrospective observational cohort study analyzed 5,049 PBE from 2,460 spring-calving cows in 8 commercial herds across 5 years (2014–2018). Cows were from convenience herds in an ongoing herd health management program that included routine reproductive pre-breeding checks conducted by University College Dublin and thus had reliable data recording. All selected herds were based in Co. Kildare, Ireland (52°60′N, 6°50′W). Cow breeds in the herds were predominantly Holstein Friesian, Jersey, or Jersey crosses. Mean (standard deviation, SD) herd size was 126 (35), and mean parity was 2.88 (1.7). Cows were typically housed in free stall slatted sheds with cubicles during the winter (November to February), and were typically at pasture from mid-February until the end of October. During the winter, lactating cows received a mixed diet consisting of grass silage (± maize silage) with concentrate supplementation in the parlor. Turn out to pasture for lactating cows was typically in mid to late February, when the main component of their diet was grass supplemented with concentrates in the parlor. Cows were milked twice per day. The mean 305-d predicted milk yield was 6,428 L/cow (SD 1,204 L). The criteria used for inclusion of cows were as follows: calving between January and May of any given year from 2014 to 2018; had their first PBE conducted at 25 DIM or more; and were assessed and assigned scores using both Metricheck® and ultrasonography. The PBE conducted on these cows were part of established routine fertility management on these farms. Cows were excluded from logistic regression analysis if they did not have adequate records of the appropriately selected risk factors.

2.3.2 Clinical Examination

As is typical of seasonal calving, pasture-based dairy systems, the majority of PBE took place before the mating start date in eligible cows (i.e., more than 25 d calved) and after the mating start in later-calving cows, once they became eligible. Examinations took place in March, April, and May each year. Each cow received 1 PBE by 1 of 3 members of the University College Dublin Health Herd Group. Examinations were conducted between 25 and 86 DIM (median 49 DIM). On the day of the PBE, all cows were identified and their BCS assigned using a 5-point system (0.25-unit increments; 1 = emaciated and 5 = obese; Edmonson et al., 1989). The perineal area of each cow was cleaned with paper towel, after which vaginal mucus samples were taken using a Metricheck® device as described by McDougall et al. (2007). Samples were assigned a mucus score (**MS**) on a scale of 0 to 3 as follows: MS0 = no or clear mucus; MS1 = clear mucus with flecks of purulent material; MS2 = mucopurulent material ≤50% white mucopurulent material, not fetid; MS3 = ≥50% purulent material or any fetid discharge (Williams et al., 2005). We defined MS2 and MS3 as positive for PVD based on previously published work (LeBlanc et al., 2002; Sheldon et al., 2006a; Dubuc et al., 2010a; Šavc et al., 2016). The

Metricheck® device was cleaned and disinfected between cows by rinsing it in a very mild (approx. 0.5-1%) chlorhexidine solution.

Immediately after the Metricheck® assessment, all cows received a transrectal reproductive examination using an ultrasound scanner (Easi-Scan 3®, 7.5-MHz linear-array transducer; BCF Technology, Mitcham, Australia). Both ovaries were examined for the presence or absence of a corpus luteum. Uterine content was scored on a scale of 0 to 4 using a modified scale based on Mee et al. (2009) and used previously by our research group (Šavc et al., 2016) and others (Carthy et al., 2014, 2016); the scale is described in Table 2.1. Uterine tract scores ≥2 were considered positive for UE, based on previously published work (Barlund et al., 2008; Mee et al., 2009; Šavc et al., 2016). All participating members received formal instructions to standardize the procedures above.

For ethical and animal welfare reasons, all cows with positive mucus or uterine tract score were recommended to be treated with closprostenol (Estrumate®, 500 μg IM; MSD Ireland, Dublin, Ireland) if a corpus luteum was present, followed by cephapirin (Metricure®, 500 mg intrauterine suspension; MSD Ireland) 72 h later; and those without a corpus luteum were recommended to be treated with cephapirin only.

For the first 6 weeks of each breeding season, all animals detected in standing estrus were bred using AI with frozen-thawed semen from bulls of proven fertility. Thereafter, stock bulls were used for breeding (for approximately 6–9 weeks). All cows were examined at least 30 d post-service to determine pregnancy status and again after 60 d post-service for final confirmation of pregnancy.

Table 2.1. Ultrasound classification of uterine tract scores (**UTS**) modified from Mee at al. (2009)

UTS	Uterine characteristics
0	No intraluminal fluid or trace volume of anechoic (black) and a spoke wheel-shaped lumen completely involuted with infolding of the endometrium.
1	Trace volume of hyperechoic (white) intraluminal fluid ≤0.2 cm in the lumen, with a spoke wheel-shaped lumen with infolding of the endometrium
2	Small volume (>0.2 cm to <0.5cm) of mixed echogenicity (grey or white) fluid in a spoke wheel-shaped lumen with infolding of the endometrium and enlarged or stellate center.
3	Moderate volume (≥0.5cm) of mixed echogenicity (grey or white) fluid in a spoke wheel-shaped lumen with enlarged or stellate center
4	Large volume (≥1 cm) mixed echogenicity (grey or white) fluid with hyperechoic particles in a circular lumen without infolding of the endometrium

2.3.3 Calving and Fertility Data

Calving and fertility data were recorded by farmers and entered into an on-farm software package (Irish Farm Computers, www.agrinet.ie). Information available on the cows included the following: date of birth; parity; dry-off dates; and predicted 305-d milk yield, fat, and protein (kg). We extrapolated 305-d yields using milk recording available at the end of lactation from each cow, obtained from the Irish Cattle Breeding Federation (www.icbf.ie). Predicted transmitting abilities for milk (kg of fat and protein) and fertility (calving interval and survival) were also obtained from the Irish Cattle Breeding Federation, and were updated several times per year using information on daughter performance data for all sires. The breeding values (predicted transmitting ability, **PTA**) for a given animal are composed predominantly of the genetic values of the cow's sire and maternal grandsire, and were based on the latest assessment conducted on March 21, 2019, for all cows. Information available on the calving period was as follows: date of calving, BCS at calving, calving assistance score using a scale of 1 to 4 (1 = normal; 2 = some assistance; 3 = considerable difficulty; 4 = veterinary assistance), stillbirth (dead within 24 h of calving), twins, calf sex, calf sire, and RFM (failure to pass fetal membranes by 24 h after calving). Other than RFM, postpartum disease information was not recorded in a standardized or consistent manner across farms and was deemed ineligible for analysis. Information on breeding included AI dates, sire, AI operator, and pregnancy test results.

2.3.4 Statistical Analysis

We carried out all data manipulation and analyses using the R statistical programming language (R Core Team, 2015). We calculated the prevalence of PVD in isolation, UE in isolation, either PVD or UE, and both PVD and UE, as a proportion of positive cows (mucus score or uterine tract score ≥2, or both) over the total cows recruited. A total of 4,952 PBE from 2,419 cows were included in the model-building process, because some examinations were missing BCS measures (47 exams; 9 cows), and some cows had no reliable PTA data available (56 exams; 32 cows) and were therefore censored. We considered PVD in isolation (model 1), UE in isolation (model 2), either PVD or UE (model 3), and both PVD and UE (model 4) as outcomes for 4 separate logistic regression models (Figure 2.1). The categorical risk factors tested were as follows: lactation number (1 to ≥ 5); breed [coded as Holstein Friesian, >11/16), Jersey (>11/16), or crossbred (>85% of which were Jersey cross)]; dry period length in days (90); calf sex; RFM; twins; stillbirth; sire type (dairy or beef and AI or stock bull); BCS at calving (thin < 2.75; ideal 2.75–3.25; fat ≥3.5); the presence of a corpus luteum at PBE; and positive RTD diagnosis by either method on the PBE of the previous year. Given the low numbers of calvings scored as a 3 or 4, we merged these levels. Continuous risk factors tested were as follows: DIM at PBE; milk production (305-d milk predicted yield in kilograms and 305-d milk predicted yield of fat and protein); and genetic merit indices for production (PTA for milk in kilograms; PTA for milk fat; PTA for milk protein) and fertility (PTA for calving interval and PTA for survival). All continuous covariates were standardized by subtracting the observation value from the mean and then dividing by the standard deviation. All standardized continuous covariates were then tested as linear and quadratic covariates.

We initially investigated individual explanatory covariates in a univariate logistic regression framework and carried them forward for multivariable regression analysis ("glmer" function in "lme4" Package; R Core Team (2015)] when $P < 0.2$. All candidate covariates for multivariable modeling were assessed for correlation. When 2 variables were highly correlated (>0.8), 1 was selected and brought forward for multivariable analysis based on the variable that resulted in the model with the lowest Akaike information criterion. Variables dropped due to collinearity were replaced in the final model to test for significance. We constructed the model using forward stepwise elimination, and variables with a significance probability of $P < 0.05$ were retained in the model. Herd, cow, and year were treated as random effects, because sampling was clustered within herd and year, and cow-level risk factors in the general Irish grazing populations were what was of interest. All 2-way interactions were assessed and retained in the model if $P < 0.05$. The models produced adjusted odds ratios (AOR), which served as an approximation of the association between risk factors and RTD.

We visualized interactions in the models by predicting the probabilities from the multivariable analysis for the variables of interest, holding all other variables at the referent and mean of the population. When the interactions were between continuous and categorical variables, we plotted out their predicted probabilities graphically for ease of interpretation; some are displayed for illustrative purposes (Figures

2.4 and 2.5). Finally, post hoc power simulations were conducted to frame the interpretations made from our study especially with regards nonsignificant variables.

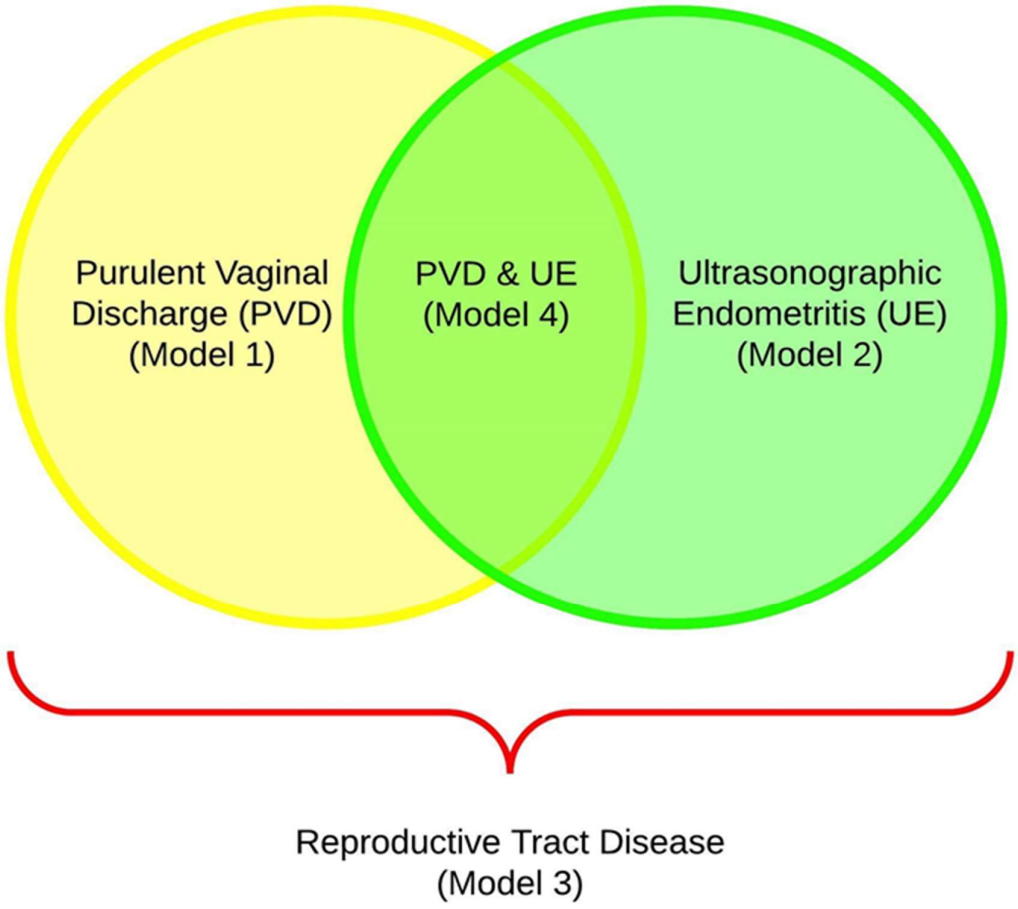

Figure 2.1. Venn diagram to outline the different risk factors models used in the present study.

2.4 Results

2.4.1 Descriptive Statistics

In total, we analyzed 5,049 PBE on 2,460 spring calving cows from 8 farms, conducted each spring period over 5 years. The approximate mean date of calving each year was February 18, and the mean DIM at PBE was 50 d, corresponding to a mean date of examination of April 9, approximately 3 weeks before the typical mating start date of the herds of May 1. We found a positively skewed distribution of DIM at first examination, summarized in Figure 2.2. The incidences of assisted calvings, RFM, stillbirth, and twins were 10.5, 1.8, 3.9, and 2.0%, respectively. The distribution of mucus score and uterine tract score for all examinations is outlined in Figure 2.3. The prevalence of RTD differed according to the diagnostic method used, and in each of the 4 models: PVD in isolation, UE in isolation, PVD or UE, and PVD and UE were 7.5, 6.7, 11.6, and 2.6%, respectively (Table 2.2). Only 35% (132/380) of cows defined as having PVD (mucus score ≥2) had uterine tract score ≥2, and only 39% (132/339) of cows defined as having UE (UTS ≥2) had mucus score ≥2. The prevalence of RTD as defined by any method was higher in the earlier DIM at examination and is outlined in Table 2.2.

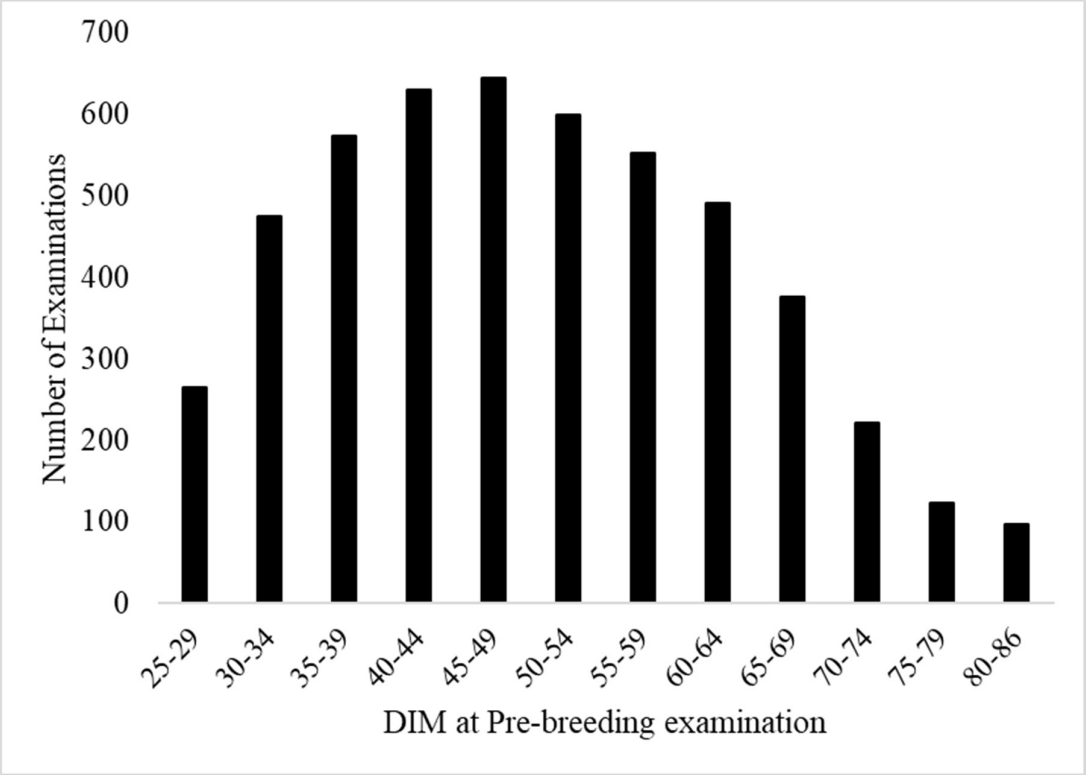

Figure 2.2. Histogram of Days in Milk (DIM) at pre-breeding examination distribution.

	UTS0	UTS1	UTS2	UTS3	UTS4	Total mucus score
MS0	3,565	482	93[3]	47[3]	14[3]	4,201
MS1	284	131	25[3]	22[3]	6[3]	468
MS2	92[3]	54[3]	31[3,4]	11[3,4]	14[3,4]	202[1]
MS3	57[3]	45[3]	18[3,4]	18[3,4]	40[3,4]	178[1]
Total UTS	3,998	712	167[2]	98[2]	74[2]	5,049

Figure 2.3. Mucus score (MS0 to MS3) and uterine tract score (UTS0 to UTS4) results for all pre-breeding examinations (n = 5,049) in the study before censoring.

Figure 2.3 Footnotes

Each cell represents the number of pre-breeding examinations with each score.

Exams in yellow = positive examinations used in the purulent vaginal discharge (PVD) model;

Exams in green = positive examinations used in the ultrasonographic endometritis (UE) model;

Exams in blue and orange = positive examinations in the PVD or UE models;

Exams in orange = positive examinations in the PVD and UE models.

Table 2.2. Prevalence of reproductive tract disease diagnosed by 2 methods [Metricheck® (Simcro, Hamilton, New Zealand) and ultrasound] used in isolation and in combination and categorized by DIM (n = 5,049 examinations).

DIM at pre-breeding examination (no. of cows)	Prevalence, % (no. positive)			
	PVD[1]	UE[2]	PVD[1] or UE[2]	PVD[1] and UE[2]
25–49 (2,589)	9.8 (253)	8.7 (224)	14.9 (387)	3.5 (90)
50–74 (2,239)	5.2 (117)	4.7 (105)	8.1 (182)	1.8 (40)
≥75 (221)	4.5 (10)	4.5 (10)	8.1 (18)	0.9 (2)
Total (5,049)	7.5 (380)	6.7 (339)	11.6 (587)	2.6 (132)

Table 2.2 Footnotes

[1]PVD = purulent vaginal discharge (positive = mucus score ≥2; mucopurulent);

[2]UE = ultrasonographic endometritis (positive = uterine tract score ≥2; >0.2 cm intrauterine fluid).

2.4.2 Model Outcomes

The final multivariable models are presented in Tables 2.3 to 2.6 and were conducted on a total of 4,952 PBE from 2,419 cows after censoring. For the multivariable analysis, only non-collinear significant factors ($P < 0.2$) were carried forward in each model. For the majority of variables, the reference category was chosen to avoid negative coefficients and odds ratios below 1. Regression coefficients and odds ratios are quoted for standardized continuous variables and their interactions, as outlined in Tables 2.3 to 2.6.

2.4.3 Risk Factors for PVD (Model 1)

The outputs from the final multivariable logistic regression for model 1 are presented in Table 2.3. The probability of PVD decreased with each day postpartum that the examination took place ($P < 0.01$). Cow factors that increased the odds of PVD were primiparity (first lactation; $P < 0.01$), Holstein Friesian breed ($P < 0.01$), having a BCS of less than 2.75 at the PBE ($P < 0.05$), and having an inferior PTA for calving interval ($P < 0.05$). Calving factors that increased the odds of PVD were giving birth to twins ($P < 0.01$) and having a calving difficulty score of ≥3 ($P < 0.01$). We found an interaction ($P < 0.01$) between dam breed and calving difficulty score, indicating that if a Jersey cow had considerable calving difficulty (score ≥3), she had a higher probability (predicted probability = 0.192) of PVD than a Holstein Friesian cow experiencing considerable calving difficulty (predicted probability = 0.065). We found an interaction ($P < 0.05$) between RFM and DIM at examination. The probability of PVD was increased if a cow with RFM was examined earlier postpartum, but this effect diminished with increasing DIM, eventually converging at approximately 55 DIM, as shown in Figure 2.4.

Table 2.3. Results from the final multivariable logistic regression (model 1) used to identify cow-level risk factors for purulent vaginal discharge (PVD)-positive cows (mucus score ≥2).

Variable	Class	No. in category	No. with PVD	Coefficient	AOR[1]	95% CI	*P*-value
Intercept				−4.05			<0.001
DIM[2]				−0.36	0.70	0.61–0.79	<0.001
Retained fetal membranes (RFM)	No	4,867	351	Referent			
	Yes	85	15	0.47	1.60	0.63–4.07	0.325
Twins	No	4,855	353	Referent			
	Yes	97	13	0.92	2.50	1.31–4.77	0.005
Calving difficulty score	1	4,433	301	Referent			
	2	371	37	−14.09	<0.01	<0.00–3.62^{E+21}	0.665
	3	148	28	2.61	13.64	4.28–43.47	<0.001
Parity	3	855	44	Referent			
	1	1,543	161	0.87	2.39	1.66–3.45	<0.001
	2	1,181	73	0.26	1.30	0.87–1.93	0.198
	4	590	36	0.14	1.15	0.72–1.83	0.563
	5+	783	52	0.14	1.15	0.75–1.77	0.520
Cow breed	Jersey	624	23	Referent			
	Crossbred	204	7	0.18	1.19	0.36–3.91	0.769
	Holstein Friesian (HF)	4,124	336	0.95	2.58	1.18–5.67	0.018
Pre-breeding examination BCS	2.75–3.25	4,537	323	Referent			
	≥3.5	70	7	0.04	1.04	0.46–2.39	0.918
	<2.75	345	36	0.50	1.64	1.09–2.46	0.016

Variable	Class	No. in category	No. with PVD	Coefficient	AOR[1]	95% CI	*P*-value
PTA calving interval[2]				0.14	1.15	1.02–1.28	0.017
DIM[2] × RFM				−1.01	0.36	0.16–0.85	0.019
HF × calving difficulty score = 3				-2.17	0.11	0.03-0.41	<0.001

Table 2.3 Footnotes

[1] AOR = adjusted odds ratio.

[2] Regression coefficients and odds ratios are for standardized continuous variables.

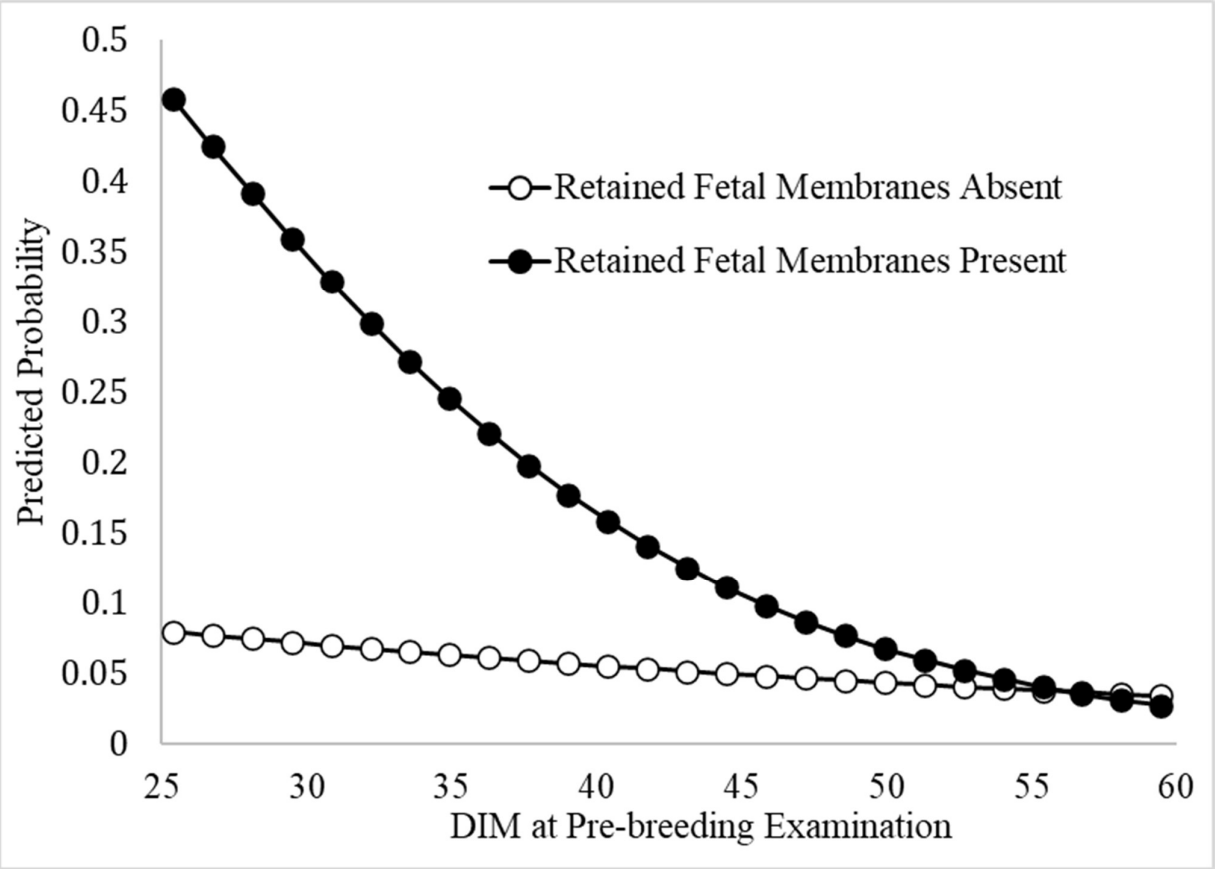

Figure 2.4. Plotted predicted probability for Model 1 (Mucus Score ≥ 2; mucopurulent) showing the shape of the interaction between days in milk and retained fetal membranes for 25 to 60 days in milk. The predicted probability of a positive score for cows that retained their fetal membranes is plotted alongside the predicted probability of a positive score for cows that did not retained their fetal membranes. Similar patterns were observed with Models 2, 3 and 4.

2.4.4 Risk Factors for UE (Model 2)

The outputs from the final multivariable logistic regression for model 2 are presented in Table 2.4. The probability of UE decreased with each day postpartum that the examination took place ($P < 0.01$). Cow factors that increased the odds of UE were multiparity (≥fifth lactation; $P < 0.01$), having an inferior PTA for calving interval ($P < 0.01$), and the absence of a corpus luteum at the PBE ($P < 0.01$). Calving factors that increased the odds of UE were giving birth to twins ($P < 0.01$) and giving birth to a calf sired by a beef breed ($P < 0.05$). We found an interaction ($P < 0.01$) between the presence of a corpus luteum on an ovary and DIM at examination. This interaction indicates that the probability of UE was higher when cows were examined earlier in milk if they had a corpus luteum present than if they did not, but the probability was much greater if a corpus luteum was not present and they were examined later in milk (Figure 2.5). Again, we found an interaction ($P < 0.01$) between cows with RFM and DIM at examination, illustrating that the probability of UE was greater in cows with RFM when examined earlier in lactation, but the probability diminished with time, eventually converging at approximately 44 DIM (similar pattern to Figure 2.4).

Table 2.4. Results from the final multivariable logistic regression (model 2) used to identify cow-level risk factors for ultrasonographic endometritis (UE)-positive cows (uterine score ≥2).

Variable	Class	No. in category	No. with UE	Coefficient	AOR[1]	95% CI	*P*-value
Intercept				−3.13			<0.001
DIM[2]				−0.50	0.60	0.51–072	<0.001
Retained fetal membranes (RFM)	No	4,867	319	Referent			
	Yes	85	13	−1.09	0.34	0.04–2.82	0.315
Twins	No	4,855	319	Referent			
	Yes	97	13	0.93	2.53	1.33–4.80	0.005
Parity	3	855	49	Referent			
	1	1,543	104	0.15	1.16	0.80–1.67	0.443
	2	1,181	61	−0.13	0.88	0.54–1.31	0.526
	4	590	39	0.08	1.09	0.69–1.70	0.719
	5+	783	79	0.52	1.69	1.14–2.48	0.008
Sire breed	Dairy	4,282	263	Referent			
	Beef	670	69	0.38	1.46	1.05–2.02	0.022
Corpus luteum present	Yes	3,641	240	Referent			
	No	1,311	126	0.45	1.57	1.22–2.05	<0.001
PTA calving interval[2]				0.15	1.17	1.04–1.30	0.006
DIM[2] × RFM				−2.51	0.08	0.01–0.47	0.005
DIM[2] × corpus luteum absent				0.52	1.69	1.13–2.18	<0.001

Table 2.4 Footnotes

[1] AOR = adjusted odds ratio.

[2] Regression coefficients and odds ratios are for standardized continuous variables.

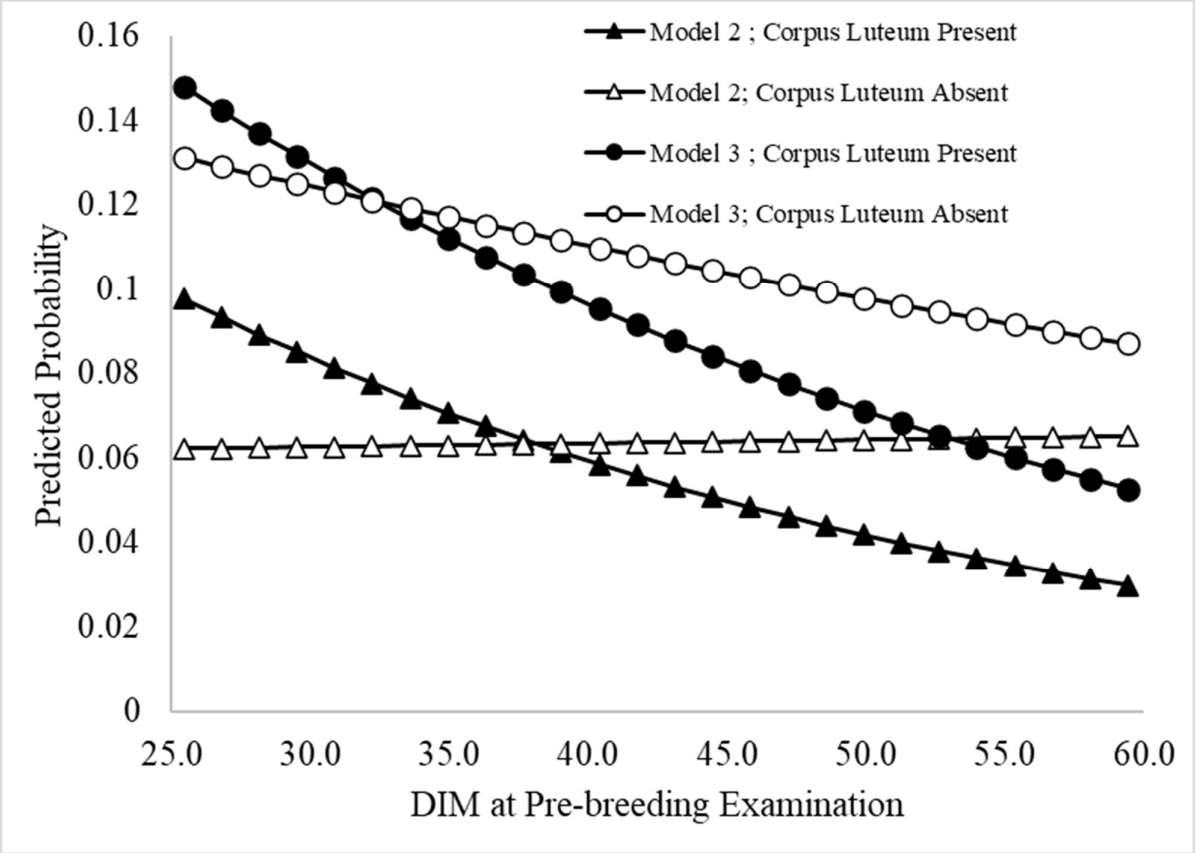

Figure 2.5. Plotted predicted probability for Model 2 (Uterine Tract Score ≥ 2) and Model 3 (Mucus Score ≥ 2 or Uterine Tract Score ≥ 2) showing the shape of the interaction between days in milk and the presence or absence of a corpus luteum at pre-breed examination for 25 to 60 days in milk. The predicted probability of a positive score for cows that had a corpus luteum present at the pre-breed examination is plotted alongside the predicted probability of a positive score for cows that did not have a corpus luteum present at the pre-breed examination.

2.4.5 Risk Factors for PVD or UE (Model 3)

The outputs from the final multivariable logistic regression for model 3 are presented in Table 2.5. The probability of PVD or UE decreased with each day postpartum that the examination took place ($P < 0.01$). Cow factors that increased the odds of PVD or UE were first and fifth lactation ($P < 0.01$), Holstein Friesian breed ($P < 0.05$), having an inferior PTA for calving interval ($P < 0.01$), and the absence of a corpus luteum at the PBE ($P < 0.01$). Calving factors that increased the odds of PVD or UE were giving birth to twins ($P < 0.05$) and having a calving difficulty score of ≥3 ($P < 0.01$). We found an interaction ($P < 0.01$) between dam breed and calving difficulty score, indicating that if a Jersey cow had considerable calving difficulty (score ≥3), she had a higher probability (predicted probability = 0.212) of PVD or UE than a Holstein Friesian cow experiencing considerable calving difficulty (predicted probability = 0.096). We found an interaction ($P < 0.01$) between the presence of a corpus luteum on an ovary and DIM at examination, as seen in model 2. This interaction again indicated that the probability of PVD or UE was higher when cows were examined earlier in milk if they had a corpus luteum present than if they did not, but the probability was much greater if a corpus luteum was not present and they were examined later in milk (Figure 2.5). Again, we found an interaction ($P < 0.01$) between cows with RFM and DIM at examination, illustrating that the probability of PVD or UE is greater in cows with RFM when examined earlier in lactation, but the probability diminished with time, eventually converging at approximately 53 DIM (similar pattern to the one seen in Figure 2.4).

Table 2.5. Results from the final multivariable logistic regression (model 3) used to identify cow-level risk factors for purulent vaginal discharge (PVD)- or ultrasonographic endometritis (UE)-positive cows (mucus score ≥2 or uterine tract score ≥2).

Variable	Class	No. in category	No. with PVD or UE	Coefficient	AOR[1]	95% CI	*P*-value
Intercept				−3.21			<0.001
DIM[2]				−0.45	0.63	0.56–0.72	<0.001
Retained fetal membranes (RFM)	No	4,867	550	Referent			
	Yes	85	20	0.34	1.40	0.56–3.48	0.471
Twins	No	4,855	553	Referent			
	Yes	97	17	0.67	1.96	1.09–3.50	0.024
Calving difficulty score	1	4,433	482	Referent			
	2	371	55	−0.62	0.54	0.07–4.19	0.555
	3	148	33	1.89	6.65	2.23–19.86	<0.001
Parity	3	855	73	Referent			
	1	1,543	230	0.70	2.01	1.49–2.71	<0.001
	2	1,181	103	0.05	1.05	0.76–1.46	0.771
	4	590	59	0.15	1.16	0.79–1.69	0.453
	5+	783	105	0.46	1.58	1.13–2.20	0.007
Cow breed	Jersey	624	43	Referent			
	Crossbred	204	11	0.04	1.04	0.41–2.63	0.933
	Holstein Friesian (HF)	4,124	516	0.64	1.89	1.01–3.55	0.046
Corpus luteum present	Yes	3,641	367	Referent			
	No	1,311	203	0.35	1.41	1.15–1.75	<0.001
PTA calving interval[2]				0.16	1.17	1.07–1.29	<0.001

Variable	Class	No. in category	No. with PVD or UE	Coefficient	AOR[1]	95% CI	*P*-value
DIM[2] × RFM				−1.28	0.28	0.12–0.65	0.003
DIM[2] × corpus luteum absent				0.27	1.31	1.06–1.61	0.006
HF × calving difficulty score = 3				−1.57	0.21	0.06–0.69	0.011

Table 2.5 Footnotes

[1]AOR = adjusted odds ratio.

[2]Regression coefficients and odds ratios are for standardized continuous variables.

2.4.6 Risk Factors for Both PVD and UE (Model 4)

The outputs from the final multivariable logistic regression for model 4 are presented in Table 2.6. The probability of PVD and UE decreased with each day postpartum that the examination took place ($P < 0.01$). The cow factor that increased the odds of PVD and UE was having an inferior PTA for calving interval ($P < 0.05$). The calving factor that increased the odds of PVD or UE was giving birth to twins ($P < 0.01$). Again, we found an interaction ($P < 0.05$) between cows with RFM and DIM at examination, illustrating that the probability of PVD or UE is greater in cows with RFM when examined earlier in lactation, but the probability diminished with time, eventually converging at approximately 42 DIM (similar pattern to the one seen in Figure 2.4).

Table 2.6. Results from the final multivariable logistic regression (model 4) used to identify cow-level risk factors for purulent vaginal discharge (PVD)- and ultrasonographic endometritis (UE)-positive cows (mucus score ≥2 and uterine tract score ≥2).

Variable	Class	No. in category	No. with PVD and UE	Coefficient	AOR[1]	95% CI	*P*-value
Intercept				−3.92			<0.001
DIM[2]				−0.43	0.65	0.53–0.80	<0.001
Retained fetal membranes (RFM)	No	4,867	120	Referent			
	Yes	85	8	−1.45	0.23	0.01–4.97	0.351
Twins	No	4,855	119	Referent			
	Yes	97	9	1.56	4.57	2.24–10.09	<0.001
PTA calving interval[2]				0.18	1.20	1.01–1.42	0.035
DIM[2] × RFM				−2.79	0.06	0.01–0.63	0.018

Table 2.6 Footnotes

[1]AOR = adjusted odds ratio.

[2]Regression coefficients and odds ratios are for standardized continuous variables.

2.5 Discussion

The prevalence of PVD in this study was similar to others recently published on PVD in grazing dairy cows when a Metricheck® device was used. Giuliodori et al. (2017), who studied the risk factors for PVD, reported a prevalence of 9.3% (213/2,303) vaginal discharge scores ≥2 (on a scale of 0–3) between 21 and 49 DIM. Similarly, McDougall et al. (2007) showed a prevalence of 9.3% (258/2,780) vaginal discharge scores ≥3 (on a scale of 0–5) in cows examined 35 d before mating start date. These findings are broadly comparable to the 9.8% (253/2,589) prevalence seen in the present study for PVD alone when cows were examined between 25 and 49 DIM. However, both of these authors included cows that had flecks of pus in clear mucus as positive PVD cases, based on their subsequent reduced reproductive performance compared with cows that had clear or no mucus. In the present study, a PVD case was defined as a cow with purulent or mucopurulent discharge, based on previous published work by this research group on a set of similar farms using the same scoring method, which indicated no significant difference between cows with flecks of pus or clear discharge (Šavc et al., 2016).

The prevalence of UE in this study was also similar to other published work. Barlund et al. (2008) showed a prevalence of 10% (22/221) endometritis when using a 90th percentile intrauterine fluid cutoff of >3 mm in cows that were examined at 28 to 41 DIM, similar to the 8.7% (224/2,589) prevalence seen in the present study when cows were examined between 25 and 49 DIM using a cutoff of >2 mm of intrauterine fluid. However, Carthy et al. (2014), who also adapted the ultrasound reproductive tract scoring method developed by Mee et al. (2009) and used it to assess risk factors for inferior uterine scores in Irish dairy cows, found a much higher prevalence (29.8%) of uterine tract score ≥2. This difference may be explained by the range in DIM at examination, which in that study was broad, ranging from 0 to 300+ DIM; not surprisingly, the earliest DIM period assessed (0 to 14 DIM) had the highest mean uterine score of 2.97 (Carthy et al., 2014), making direct comparison with this study difficult. The prevalence of both PVD and UE was 3.5% (90/2,589) between 25 and 49 DIM in the present study, and as far as we are aware, this has not been stated in any other previous published work. Dubuc et al. (2010a) found that the prevalence of both cytological endometritis (≥6% PMN) and PVD (≥mucopurulent) was 5.8% (61/1,044) at 35 DIM, which was higher than the present study but was likely due to the poor sensitivity of ultrasound to identify cytological endometritis, a fact that has been noted by others (Barlund et al., 2008).

The shared risk factors across all 4 models were DIM at time of examination, the interaction between DIM at examination and RFM, twinning, and a cow's inferior PTA for calving interval. The effect of DIM was significant in this study; the earlier the PBE was performed, the higher the likelihood of testing positive with either method alone or both in combination. This finding has been noted by others (Griffin et al., 1974; Gautam et al., 2010; Giuliodori et al., 2017), and it is most likely because of the high clinical self-cure rates that tend to be seen in cows with RTD; some publications report clinical self-cure rates ranging from 57 to 77% (Dubuc et al., 2010b; Gautam et al., 2010; Giuliodori et al., 2013a; Giuliodori

et al., 2017). This apparent self-cure may be a true complete cure or a change to the subclinical state. This clinical self-cure rate also depends on the time at first diagnosis and the severity of the condition; those with higher scores have lower odds of self-cure or medical cure following treatment (Burke et al., 2010; Giuliodori et al., 2017).

Retained fetal membranes are a well-established risk factor for RTD (Kim and Kang 2003; Potter et al., 2010). Their presence delays involution and restoration of the endometrium, as well as maintaining the patency of the cervix, facilitating an entry point and a suitable environment for bacterial overgrowth (Sheldon et al., 2008). In the present study, the apparent prevalence of RFM was 1.8%, which is lower than expected when compared with the prevalence of 4.06% (Mee, 1991) and more recently 2.3% (Buckley et al., 2007) found by others studying Irish grazing herds; this finding may have been due to inefficient recording. Despite the likely under-reporting, the significant interaction between RFM and DIM found in all models in the present study is an interesting finding. This interaction suggests that the probability of a positive diagnosis by either Metricheck® or ultrasound is much higher in a cow who has RFM when examined early in milk than if she is examined later, when the probability of a positive diagnosis converges with that of a cow without RFM (Figure 2.4). This stresses the importance—especially in compact calving grazing systems—of targeting examinations earlier in milk to those who have RFM, particularly if these cows are also later calving and do not have the same amount of time to recover before breeding commences.

Twinning is a well-established risk factor for the development of RTD (Bell and Roberts, 2007; Dubuc et al., 2010b) and was significant across all 4 models. Twinning likely contributes to RTD through increased risk of assistance or dystocia (Mee et al., 2011), RFM (Nielen et al., 1989), and metabolic issues (Markusfeld, 1987; Nishida et al., 1997). Knowledge of twinning as a risk factor is important not only to identify cows that are more at risk of contracting RTD but also to implement preventative measures to mitigate that risk. Diligent monitoring and skilled assistance at calving (Mee, 2004), early dry-off due to shortened gestation (Nielen et al., 1989), and minimizing BCS loss in this cohort over the dry period (Bell and Roberts 2007) have been suggested and may play a role in reducing RTD in this cohort of cows.

The predicted transmitting ability or the genetic value for calving interval that will be transmitted to the cow's offspring was associated with RTD across all 4 models. Cows with higher values for calving interval had greater odds of being diagnosed with RTD, regardless of the diagnostic method. Heritability estimates for RTD are generally low and vary across populations from 0.02 to 0.03 in Irish and Canadian cows (Koeck et al., 2012; Carthy et al., 2015) to 0.07 in the United States (Zwald et al., 2004). Interestingly, Carthy et al. (2015) and Fitzgerald et al. (2014), both using a similar ultrasound scoring system, found that an inferior uterine environment was associated with traditional reproductive traits such as calving interval, and that selection for superior genetic merit for the traditional reproductive traits was associated with favorable outcomes in these traits; this study supports those findings. To our

knowledge, this is the first time the association has been shown for PVD in an Irish context, but this should not be surprising, because others have reported that estimates for the different assessments of RTD (metritis, PVD, and endometritis) are similar, and the genetic correlations between them do not differ (Koeck et al., 2012).

Some authors have described a quadratic association between parity and RTD; primiparous cows and cows in their third lactation or greater are more likely to develop metritis than cows in their second lactation. (Markusfeld, 1984; Gröhn et al., 1990; Bruun et al., 2002). This finding was supported by the present study in model 1, which found that primiparous cows were at higher odds of PVD than multiparous cows, as noted by others (Potter et al., 2010; Giuliodori et al., 2017.) Model 2 showed that older cows (≥fifth lactation) were at greater risk of having UE, a finding that has also been described by others (Carthy et al., 2014). Model 3 showed this quadratic association, because both parity groups were at risk when either PVD or UE was considered RTD. Primiparous cows are more likely to develop dystocia and genital tract damage as a result (Meyer et al., 2001; Mee et al., 2011). This genital tract damage likely results in impaired cervical patency and resultant bacterial colonization of the uterus; however, it is certainly possible that in a proportion of these cases the PVD originates from cervical or anterior vaginal inflammation and not endometrial inflammation, a conclusion shared by others (Deguillaume et al., 2012). This may help to explain why primiparae are at greater risk of PVD and not UE, as seen in this study. Others have found that endometritis and ultrasound tract scoring deteriorates by parity number (Kim and Kang 2003; Carthy et al., 2014), as seen in the present study, and may be explained by poor vulval angle (Potter et al., 2010), urovagina (Gautam and Nakao, 2009), and impaired bacterial clearance in older cows (≥fifth lactation; Gilbert et al., 1993) which are all risk factors for RTD.

Calving difficulty score (≥3) was a significant risk factor for PVD alone (model 1) and PVD or UE (model 3), but not for UE alone (model 2) or a combination of PVD and UE (model 4). The finding of assisted calvings or dystocia as a risk factor for PVD was not surprising and has been reported by many others (Dubuc et al., 2010b; Potter et al., 2010; Giuliodori et al., 2013, 2017). As in the present study, there have been conflicting reports about the association between dystocia and UE. Carthy et al. (2014) found that cows with dystocia in their previous calving had inferior ultrasonographic uterine scores, in contrast to this study. However, Dubuc et al. (2010b) and Cheong et al. (2011) both failed to find dystocia as a risk factor for subclinical endometritis, more in agreement with the present study.

Breed was a significant risk factor for PVD alone (model 1) and PVD or UE (model 3), but not for UE alone (model 2) or a combination of PVD and UE (model 4). In both models 1 and 3, Holstein Friesian cows were at greater risk of having PVD alone or a combination of PVD or UE than Jersey cows, in contrast to other studies in grazing cows, which found no difference (McDougall et al., 2007; Carthy et al., 2014). More interesting, however, was the interaction we observed between calving difficulty and breed, indicating that a Jersey cow with a difficult calving (score ≥3) had a much greater likelihood of developing endometritis than a Holstein Friesian cow with a difficult calving. This interaction seems to

imply that when difficult calvings occur in Jerseys, these cows are more likely to experience genital tract trauma, inflammation, and endometritis as a result. More focused efforts, such as careful sire selection and early dystocia intervention, may help to reduce RTD in this cohort.

Cows who gave birth to beef-sired calves were at increased risk of UE in model 2, but not in the other models. A wide array of AI and stock bull sire breeds were used on farms in this study, from dairy breeds (Holstein Friesian and Jersey) to continental beef breeds (Charolais, Limousin, and Belgian Blue) and early-maturing beef breeds (Angus and Hereford), and were broadly categorized as beef or dairy sires. Mee et al. (2011) has shown that the likelihood of calving assistance or dystocia was greater for males sired by Charolais sires in Holstein Friesian cows in Ireland, and this may explain why beef-sired calves play a role in increasing the odds of developing UE seen in cows in this study. Use of a stock bull was significant in the univariable analysis, but not in any final multivariable model. In this study, only 5% of AI sires were beef breeds, and 66% of stock bull calves born were from beef sires. Because the use of beef stock bulls allows farmers to produce crossbred calves whose carcasses are more valuable than those of purebred dairy calves once replacements are bred (Wolfová et al. 2007), this finding would imply that use of easier-calving beef stock bulls may help reduce the incidence of UE.

Thinner cows (BCS < 2.75) at PBE had greater odds of PVD than cows with ideal BCS in model 1, but not in the other models. This thinner group of cows most likely included 2 subsets: those that had lost excessive condition post-calving, and those that were thin at calving. This finding indicates the role that peripartum negative energy balance likely plays in the development of RTD. This finding has also been reported by others with high prepartum fatty acids and high postpartum BHB (Dubuc et al., 2010b; Cheong et al., 2011; Giuliodori et al., 2013a), BCS loss post-calving (Giuliodori et al., 2017), and low BCS at calving (Hoedemaker et al., 2009; Dubuc et al., 2010b), all having being shown to increase the odds of PVD and subclinical endometritis. It should be noted that we found a trend for fat cows (BCS >3.5) at calving to be at higher risk of RTD in our study, but this was not seen in the final multivariable models and may have been due to a lack of statistical power in the models because of the low overall prevalence of fat cows at calving in our study.

The absence of a corpus luteum at PBE increased the risk of UE (model 2) and PVD or UE (model 3), and a similar pattern has been found by others (Senosy et al., 2009). Bacterial contamination of the endometrium is associated with reduced function of the dominant ovarian follicle (Sheldon et al., 2002b; Williams et al., 2007) and delayed resumption of cyclicity (Opsomer et al., 2000), which may explain this finding. However, we found a significant interaction between DIM and the presence of a corpus luteum at PBE in both models (Figure 2.5). The interaction implied that cows with a corpus luteum present and examined at approximately <37 DIM (model 2) and <32 DIM (model 3) had a higher likelihood of a positive diagnosis than those without a corpus luteum (Figure 2.5). However, beyond approximately 32 to 37 DIM, the presence of a corpus luteum at PBE reduced the likelihood of a positive diagnosis (Figure 2.5). Some have reported that cows with an early resumption of cyclicity (before 21

DIM) had lower odds of subclinical endometritis at 49 DIM than cows that had not resumed cyclicity by 49 DIM (Galvão et al., 2010). Others have found that ovulatory status before the diagnosis of subclinical endometritis (at 42 DIM) was not a risk factor, but that cows with a prolonged resumption of cyclicity (>56 DIM) were more likely to have endometritis as a preceding condition (Burke et al., 2010). In the present study, reproductive exams were taken at different time points in each cow, corpus luteum status and diagnosis of RTD were determined at PBE, and the earliest exam took place at 25 DIM. Therefore, it was not possible to determine whether early ovulation status influenced the risk of developing endometritis, even though cows with a corpus luteum at <35 DIM appeared to have a higher predicted probability of a positive diagnosis. Likewise, is it not possible to say if these cows would have tested positive if examined later in milk. However, it is possible to infer that cows with no evidence of cyclical or estrus activity >42 DIM have higher odds of a positive diagnosis of UE and should be targeted for examination and intervention in seasonal calving systems, a finding noted by others (McDougall et al., 2007).

Many risk factors for RTD are well established, and a broad selection of these risk factors were tested in this study. Previously important risk factors such as calf sex (Potter et al., 2010) and stillbirth (Markusfeld, 1984; Potter et al., 2010) were not found to be significant. In a UK study on Holstein Friesian cows in an indoor system (Potter et al., 2010), the birth of a male calves was a risk factor for PVD, but in our study, male sex appeared to have no effect. One potential reason for this could be that our study lacked sufficient statistical power. However, we conducted a post hoc power simulation based on the differences noted for calf sex stated by Potter et al. (2010). From this analysis we found that given the effect of male sex noted in that study population, our statistical power was 0.91 given our sample size.

In the same study (Potter et al., 2010), stillbirth was also significant in the final model, and although stillbirth was significant on the univariate analysis in our study, it was not significant in any final multivariable models. This finding may have been due to differences in definitions of stillbirth (dead or alive at birth versus dead within 24 h), or that our study was underpowered. In this case, we calculated a power of 0.71 for stillbirth based on our post hoc power analysis. This finding may also have been due to the fact that dystocia and stillborn risk in the UK has been attributed to “holsteinization” (McGuirk et al., 1999), which may not be as profound in Ireland, or even that some have found stillbirth in Holstein Friesian primiparae in Ireland to be increasingly unrelated to dystocia (Mee, 2006). All of this emphasizes the importance of testing and validating different risk factors in diverse cow populations.

Peripartum disease conditions such as metritis (Dubuc et al., 2010b), ketosis (Dubuc et al., 2010b), and hypocalcemia (Whiteford and Sheldon et al., 2005), which have all have been shown to be risk factors for RTD, were not tested in this study and cannot be ruled out as important risk factors. These will require future evaluation in future studies into RTD in grazing Irish dairy cows.

2.6 Conclusions

Even though PVD and UE are imperfect diagnostic tests, they remain the most practical cow-side options for RTD. Both methods of diagnosis share many common cow-level risk factors, which include DIM at PBE, RFM, twins, and cow genetics. Other risk factors that are specific to other diagnostic methods are considerable calving difficulty, parity, BCS, dam or sire breed, and the cow's cyclical activity. This may be because these diagnostic methods detect different manifestations of RTD in some instances. However, because both PVD and UE in isolation have been shown to have detrimental effects on subsequent reproductive performance, all of these findings should be considered for preventative advice and identifying at-risk cows

Chapter 3

Reproductive tract disease in Irish grazing dairy cows: Retrospective observational study examining its association with reproductive performance and the accuracy of two diagnostic tests.

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3.0. Abbreviations used in this chapter

Se: Test sensitivity

Sp: Test specificity

HR: Hazard Ratio

BPI: Bayesian probability intervals

PTAs: Predicted transmitting abilities

MSD: Mating Start Date

EESD: Earliest eligible service date

DO: Days open

MCT: Misclassification cost term

RTD: Reproductive Tract Disease

PBE: Pre-breeding examination

PVD: Purulent vaginal discharge

- The scoring system in this Chapter is the same as in Chapter 2 but mucous score (**MS**) is replaced with PVD score
- It still is based on Williams et al. (2005)
- Please see Table 1.3 for a detailed description, although it is also detailed in the text
- Please note that PVD score is synonymous with MS score as used Chapter 2
- This has been left as such to keep consistency with published and submitted manuscripts.

UE: Ultrasonographic endometritis

- The scoring system in this Chapter is the same as in Chapter 2 but uterine tract score (**UTS**) is replaced with UE score
- It is still based on Mee at al. (2009).
- Please see Table 2.1 for a detailed description, although it is also detailed in the text.
- Please note this is synonymous with UTS score as used Chapter 2.
- This has been left as such to keep consistency with published manuscripts.

3.1 Abstract

The detection of reproductive tract disease (RTD) three weeks postpartum is important due to its' impact on subsequent reproductive outcome. Numerous methods for the diagnosis of RTD are described, some of which are more practical and instantaneous in terms of diagnosis. Two of these methods involve identification of purulent vaginal discharge (PVD) or evidence of ultrasonographic uterine changes indicative of endometritis (UE). The objectives of our retrospective observational study were 1) to assess the association of PVD or UE score at the pre-breeding examination (PBE) on the hazard of pregnancy within the subsequent breeding season; 2) to determine the test sensitivity (Se) and specificity (Sp) at the point of sampling of both tests using a Bayesian latent class model and 3) to determine the effect of varying positivity thresholds on test accuracy. To achieve these, we analyzed an initial dataset of 5,049 PBEs from 2,460 spring-calved cows in 8 herds between 2014 and 2018. Each PBE was conducted once between 25-86 DIM. At each PBE, vaginal discharge was obtained with a Metricheck device while transrectal ultrasonography assessed uterine content. PVD was scored on a scale of 0-3 depending on discharge character and UE was scored on a scale of 0-4 depending on the presence and consistency of intraluminal fluid. Cows with scores of ≥ 2 in either test had received treatment.

Fertility data was available from 4756 PBEs after data exclusion. The association between PVD or UE score at the PBE and subsequent hazard of pregnancy was analyzed using Cox proportional hazards model. Cows with PVD score 2 or 3 were less likely to conceive than cows with PVD score 0 (score 2 Hazard Ratio (HR)=0.74;95%CI:0.59-0.94; score 3 HR=0.65;95%CI:0.51-0.84). Cows with UE score 1, 2, 3 or 4 were less likely to conceive than cows with UE score 0 (score 1 HR=0.82;95%CI:0.73-0.93; score 2 HR=0.79;95%CI:0.62-1.00; score 3 HR= 0.43;95%CI:0.43-0.90; score 4 HR=0.39;95%CI:0.26-0.58).

To determine the Se and Sp of PVD or UE scoring for diagnosis of RTD at the time of PBE, a Bayesian latent class model was fitted on 2,460 individual cow PBEs. Flat priors were used for the Se and Sp of UE, while informative priors for PVD Se (mode = 65%, 5th percentile = 45%) and Sp (mode = 90%, 5th percentile = 80%) and RTD prevalence (mode = 20%, 5th percentile = 10%) were used. Posterior estimates (median and 95% Bayesian probability intervals; BPI) were obtained using 'rjags' (R-Studio). The optimal test thresholds (PVD and UE score ≥1) were selected by assessing the impact of different thresholds on test estimates and using a misclassification cost analysis. Based on these, median (95% BPI) Se for PVD and UE score ≥ 1 was 44%(29-60%) and 67%(33–100%), respectively. Median Sp for PVD and UE score ≥1 was 90%(86–93%) and 91%(86–93%), respectively. Higher scores in both tests were associated with impaired fertility and UE scoring with a threshold of ≥1 had the highest test Se and Sp estimates but test Se was conditional on DIM at when the PBE occurred.

3.2 Introduction

Endometritis is defined as the inflammation of the endometrium which occurs after the first three weeks post-partum (Sheldon et al., 2006). It has profound effects on the reproductive performance of the dairy cow by reducing submission (i.e., likelihood of being submitted for an insemination) and conception (i.e., likelihood of becoming pregnant to an insemination) risk which leads to increased days open (i.e., time to pregnancy) (LeBlanc et al. 2002; Giuliodori et al., 2013; Giuliodori et al., 2017). Any condition that prolongs days open are of particular importance in seasonal calving pasture-based cows where time to conceive is limited by the restricted breeding season (typically 12 weeks) in such systems (McDougall, 2006).

Over the years, the definition of what constitutes 'endometritis' after the first three weeks post-partum has evolved and changed, as new diagnostic techniques have been introduced and the understanding of the effects on subsequent reproductive performance has improved. Diagnostics used to detect endometritis include visual assessment of cervical discharge by vaginoscopy (Leutert et al., 2012), examination of the vaginal discharge obtained with a gloved hand (Sheldon et al., 2002a; Williams et al., 2005) or a Metricheck device [Simcro, Hamilton, New Zealand; (McDougall et al., 2007)], ultrasonographic assessment of uterine contents (Mee et al., 2009), biochemical tests (e.g. leukocyte esterase tests) on vaginal discharges or uterine lavages (Denis-Robichaud and Dubuc, 2015), cytological quantification of the proportion of polymorphonuclear cells in samples obtained from cytobrushing or uterine lavage (Barlund et al., 2008), microbiological culture of uterine swabs (McDougall et al., 2011) and endometrial biopsies (Bogado Pascottini et al., 2016). These differing diagnostic methods have led to the subcategorization of endometritis into clinical (i.e., based on visual assessment of reproductive tract discharges) and subclinical (i.e., based on cellular changes assessed with microscopic examination of endometrial smears/biopsies) states (Sheldon et al., 2006). More recently, some authors (Dubuc et al., 2010) have suggested to retitle the term clinical endometritis as purulent vaginal discharge (**PVD**). The same author has shown that there is poor agreement between PVD and endometritis based on cytology when both are assessed simultaneously (Dubuc et al., 2010). Therefore, the argument in favor of this re-classification is strong, considering that when the diagnosis is based on retrieving discharge from the anterior vagina using a gloved hand or Metricheck device, the operator is failing to determine the precise origin of the discharge (i.e., is it from an endometritis, cervicitis, vaginitis or combined inflammation of all these structures) and hence the term PVD may be more appropriate (Dubuc et al., 2010, de Boer et al., 2014). Finally, pyometra, which is defined as the accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix, is deemed by many as a clinical sequela of endometritis (Sheldon et al., 2006). This condition is unlikely to yield purulent discharge, but it should be clinically detectable by transrectal ultrasonography as fluid accumulation of varied volume and echodensity in the uterine lumen (Sheldon et al., 2006). For the purpose of this manuscript, the term "reproductive tract disease" (**RTD**) will be used as an all-

encompassing term to describe both clinical/subclinical endometritis, pyometra and PVD occurring after three weeks postpartum.

A systematic review by de Boer et al., (2014) highlighted the challenges facing the reporting of studies on the diagnostics techniques for RTD. They concluded that the quality of future research would be improved by following a pre-defined checklist, tying diagnostic criteria to objective measures of reproductive outcome and by using enhanced statistical approaches that do not rely on standard reference tests when assessing test sensitivity (**Se**) and specificity (**Sp**) (de Boer et al., 2014). Latent class models can be used in situations where no standard reference test exists (van Smeden et al., 2014) and thus are particularly useful in analyzing the diagnostic tests for RTD due to the aforementioned challenges in diagnosis. In Bayesian analyses, all parameters are considered unknown and their values are modelled using probability distributions which are often informed based on previous research data (Joseph et al., 1995). Thus, in the context of many diagnostic test studies such as the case of RTD, applying a Bayesian approach to latent class models, allows for various parameters (e.g., prevalence, test Se, Sp) to be estimated (Kostoulas et al., 2017). These estimates are conditional on both current observed and previous research data about the disease and/or diagnostic tests and thus provide useful inferences for researchers.

Recently, this approach has been applied to RTD by some authors (Arango-Sabogal et al., 2019; McDougall et al., 2020) and this has considerably enhanced our understanding of the prevalence and the test Se and Sp of common cow-side diagnostics for RTD. Arango-Sabogal et al. (2019), using a Bayesian latent class model estimated a median within-herd prevalence of RTD of 47.9% (95% Bayesian probability interval (**BPI**); 39.0-56.7%) at the moment of sampling in population of Canadian dairy cows sampled at between 28-42 DIM. McDougall et al. (2020), estimated a median within-herd prevalence of 22.9% (95% BPI: 10.4-40.1%) in population of New Zealand seasonal dairy cows with sampling conducted on an average of 47 DIM. In terms of diagnostic tests evaluated (i.e., PVD scoring with Metricheck, endometrial cytology and leukocyte esterase tests), both studies determined that PVD scoring had the highest test Se and Sp, although test Se was not particularly high for any test.

Although many studies have illustrated that ultrasonographic changes in the uterus indicative of endometritis (**UE**) are useful for assessing RTD, and practical in predicting reproductive outcome (Mee et al., 2009; López-Helguera et al., 2012; Šavc et al., 2016), few have determined test Se and Sp at the point of sampling (Barlund et al., 2008; Meira et al., 2012). The two latter studies determined test Se and Sp of ultrasound findings (i.e., intrauterine fluid quantity or endometrial thickness) relative to cytological endometritis determined by cytobrush sampling (Barlund et al., 2008; Meira et al., 2012) or histopathological examinations from uterine biopsy (Meira et al., 2012). However, to the best of our knowledge, the diagnostic accuracy (Se and Sp) of ultrasonographic scoring systems at the time of sampling has not been assessed using a Bayesian latent class model.

Therefore, based on the guidelines on the reporting of research on RTD (de Boer et al., 2014), the objectives of our retrospective observational study were 1) to assess the association of PVD or UE scores at the PBE on the hazard of pregnancy within the subsequent breeding season of Irish spring calving pasture based dairy cows; 2) to determine the Se and Sp of PVD and UE scoring at the time of the PBE for diagnosis of RTD using a Bayesian latent class model and 3) to determine the effect of varying positivity thresholds of both tests on overall test accuracy.

3.3 Materials and Methods

3.3.1 Study Dataset

The retrospective dataset used was the same as per Kelly et al. (2020) where risk factors for RTD were assessed. It comprised the results of 5,049 pre-breeding examinations (**PBEs**) from 2,460 spring-calving cows in 8 commercial seasonal dairy herds that were conducted each spring over a 5-year period (2014–2018). PBEs were clustered within cows and cows clustered within each of the 8 herds. A cow received a single PBE prior to breeding each year and there was a mean of 2.05 PBEs per cow (range 1-5) in the dataset. The cow was the study unit on which the PBE was applied with some individual cows having multiple PBEs conducted over multiple years comprising the dataset.

3.3.2 Animals and Herds

The sampled cows originated from conveniently selected dairy herds located in Co. Kildare, Ireland (52°60′N, 6°50′W). Each of the herds were enrolled in an ongoing herd health management program operated by Farm Animal Clinical Studies Section, School of Veterinary Medicine (University College Dublin) and thus, had reliable data recording. Cow breeds in the herds were predominantly Holstein Friesian, Jersey, or Jersey crosses. Mean (SD) herd size was 126 (35), and parity was 2.88 (1.7). Cows were typically housed in freestall slatted sheds with cubicles during the winter (November to February), and were typically at pasture from mid-February until the end of October. During the winter, lactating cows received a mixed diet consisting of grass silage (± maize silage) with concentrate supplementation in the parlor. Turn out to pasture for lactating cows was typically in mid to late February, when the main component of their diet was grass supplemented with concentrates in the parlor. Cows were milked twice per day. The mean 305-d predicted milk yield was 6,428 L/cow (SD 1,204 L). All cows in the dataset had calved in spring (i.e., between January and May) in any given year.

3.3.3 Pre-breeding examination

As is typical of seasonal calving, pasture-based dairy systems, the majority of PBEs took place before the mating start date in eligible cows (i.e., more than 25 d calved) and after the mating start in later-calving cows, once they became eligible. The PBE conducted on these cows were part of established routine reproductive management on these farms. Examinations took place in March, April, May and June each year. A cow received a single PBE each year by 1 of 3 members of the University College Dublin Health Herd Group and all members were formally trained to standardize the sampling procedure. Examinations were conducted between 25 and 86 DIM (median 49 DIM) and consisted of BCS assessment on a 5-point scale (Edmonson et al., 1989), appraisal of vaginal discharge obtained by a Metricheck device and ultrasound examination of reproductive tract (the two latter assessments are outlined in further detail below).

3.3.4 Purulent vaginal discharge (PVD) examination

After the perineal area of each cow was cleaned with paper towel, vaginal discharge was obtained by using a Metricheck device as described by McDougall et al. (2007). Once the sample was obtained it was visually assessed by the operator. Vaginal discharge was scored on a scale of 0 to 3 as follows: PVD score 0 = no or clear mucus; PVD score 1 = clear mucus with flecks of purulent material; PVD score 2 = mucopurulent material <50% white mucopurulent material, not fetid; PVD score 3 = ≥50% purulent material or any fetid discharge (Williams et al., 2005) (see Table 1.3).

3.3.5 Ultrasonographic examination for detection of endometritis (UE)

The ultrasonographic examination took place immediately after the vaginal discharge assessment, and it was conducted using a 7.5-MHz linear-array transducer (Easi-Scan 1; IMV imaging, Bellshill, UK). During the ultrasonographic examination, ovaries were examined for the presence or absence of a corpus luteum, and the entire uterus was assessed in cross section for the presence of intraluminal fluid of varying degrees. Ultrasonographic examination for the detection of changes associated with endometritis the uterus (**UE**) was scored on a scale of 0 to 4, modified from Mee et al. (2009), as follows: UE score 0 = No intraluminal fluid or trace volume of anechoic (black) and a spoke-wheel-shaped lumen; UE score 1 = Trace volume (≤2 mm) of hyperechoic (white) intraluminal fluid in a spoke-wheel-shaped lumen with infolding of the endometrium; UE score 2 = Small volume (>2 to <5 mm) of intraluminal fluid of mixed echogenicity (grey or white); UE score 3 = Moderate volume (≥5 to <10 mm) of intraluminal fluid of mixed echogenicity (grey or white); UE score 4 = Large volume (≥10 mm) of intraluminal fluid of mixed echogenicity (grey or white) (see Table 2.1). An example of these grades is also included in the supplementary materials (Section 3.7, Figure 3.9).

After the completion of both examinations, for ethical, animal welfare and commercial reasons, all cows with a PVD score of ≥ 2 or UE score of ≥ 2 were recommended to be treated with closprostenol (Estrumate, 500 µg IM; MSD Ireland, Dublin, Ireland) if a corpus luteum was present, followed by cephapirin (Metricure, 500 mg intrauterine suspension; MSD Ireland) 72 hours later; while those without a corpus luteum were recommended to be treated with cephapirin only.

3.3.6 Breeding management and pregnancy diagnosis

Each of the 8 farms operated a typical seasonal breeding season which consisted of a period of approximately 6 weeks of artificial breeding commencing on a herd specific calendar date ('mating start date'; **MSD**) followed by a period of natural service with stock bulls. During the artificial breeding period, all cows detected in standing estrus were bred using AI with frozen-thawed semen from bulls of proven fertility. During the natural breeding period, bulls were introduced to all cows in the herd and it varied from 6-9 weeks in duration after which all breeding was ceased ('mating end date'). Calving, fertility, and some descriptive cow data were recorded by farmers and entered into an on-farm software

package (Irish Farm Computers, www.agrinet.ie) and this was uploaded to Irish Cattle Breeding Federation (www.icbf.ie) where further genetic merit and production data were updated and made available. Recorded calving data available for analysis included date of calving, BCS at calving, calving assistance score using a scale of 1 to 4 (1 = normal; 2 = some assistance; 3 = considerable difficulty; 4 = veterinary assistance), stillbirth (dead within 24 h of calving), twins, calf sex, calf sire, and RFM (failure to pass fetal membranes by 24 h after calving). Fertility data available for analysis included date of service; type of serve (artificial insemination (AI) versus farmer recorded bull serve); sire used; AI operator and pregnancy status following each service which was based on subsequent pregnancy diagnosis (see below). Descriptive cow data available for analysis included date of birth; parity; dry-off dates; predicted 305-d milk yield, fat, and protein (kg) and predicted transmitting abilities (**PTAs**) for milk (kg of fat and protein) and fertility (calving interval and survival).

For pregnancy diagnosis, all cows were examined by 1 of the same 3 operators at routine visits in the months of June, July, August and September each year. At each visit, cows that were at least 30 days post-service were examined to determine pregnancy status. Additionally, all cow determined pregnant from their initial pregnancy diagnosis were re-examined at least 60 days post-service for final confirmation of pregnancy as part of routine management. The 'days-in-calf' or stage of gestation was estimated by the operators by measuring crown rump length in early gestation and head length in later gestation. When stage of gestation on ultrasound examination closely matched that of a recorded service date, this was taken as the date of conception. When stage of gestation did not match that of a recorded service date or there was no service date recorded for cow (i.e., in the case of bulls serves that the farmer failed to record), the estimated stage of gestation was deducted from the date of pregnancy diagnosis to estimate a date of conception for the farmer.

3.3.7 Data manipulation and statistical analysis software

All data manipulation, visual presentation and statistical analysis was conducted using R statistical programming (R Core Team, 2015) using 'tidyverse'(Wickham et al., 2018), 'survival' (Therneau et al., 2021) and 'rjags' (Plummer et al., 2019) packages. The Bayesian latent class analysis section of this manuscript was written along the standards for the reporting of diagnostic accuracy studies that use Bayesian latent class models (Kostoulas et al., 2017) and the checklist is included in the supplementary materials (Section 3.7, Table 3.10).

3.3.8 Descriptive statistics of subsequent reproductive outcome

Data were excluded from the reproductive outcome analysis due to incomplete records for the following reasons, missing BCS measures (n = 41 exams), missing PTA data on certain cows (n = 56 exams) and missing fertility data (n = 196 exams). Thus, complete data was available on 4756 PBEs on a total of 2338 cows on the 8 farms over the 5-year period and all cows in the data set were eligible to become pregnant over each breeding period. The results of each PBE conducted on the cow (i.e. the study unit)

within each year were used to assess the associations between PVD or UE score at the PBE, and other explanatory variables, on subsequent reproductive outcome in the breeding season.

For descriptive summary statistics, commonly used seasonal reproductive performance outcomes were calculated for each cow that had a PBE conducted in each given year (n= 4756 PBEs). These reproductive performance outcomes included the proportion of cows submitted for AI within 24 days of their earliest eligible service date (**EESD**), the first service pregnancy per AI, days open (**DO**) in the breeding season, and the proportion of pregnant cows within 6 weeks after MSD. The exact definitions and methods of calculation for these aforementioned parameters are outlined in further detail in Table 3.1. To control for later calving cows in some of these calculations (i.e., proportion submitted for AI within 24 days of EESD and DO), the EESD was defined as the herd MSD for cows ≥ 24 DIM at MSD while for cows < 24 DIM at MSD, the EESD was equal to the calving date plus 24 days. Unadjusted means and 95% confidence intervals were calculated for descriptive purposes and each of these reproductive performance outcomes and are illustrated in Table 3.2.

Table 3.1. Commonly used seasonal fertility parameters used within the manuscript to describe reproductive performance in the seasonally calving pasture-based herds in the study and their methods of calculation.

Terms used in the manuscript	Definition	Calculation
Earliest Eligible Service Date (EESD)	Date at which a cow becomes eligible for breeding.	For cows ≥24 DIM at Mating start date (MSD) = MSD in the herd For cows <24 DIM at MSD = Calving Date + 24 days
24 day submission rate from EESD	Proportion of cows submitted for service within the first 24 days from Earliest eligible Service Date.	(Number of cows submitted for AI within EESD + 24 days ÷ Total number of cows in data set) × 100
First Service Pregnancy per AI	Proportion of cows pregnant to first artificial insemination.	(Number of cows pregnant to first AI ÷ Total number of first AIs) × 100
Days Open (DO)	Numbers of days from EESD to conception for those conceiving and EESD to end of breeding season for those non-conceiving.	For pregnant cows; (Date of Conception – EESD) + 1 For non-pregnant cows; (Herd mating end date – EESD) + 1
6-week in-calf rate	Proportion of cows conceiving within the first 6 weeks from MSD	(Number of cows pregnant within MSD + 42 days ÷ Total number of cows in data set) × 100

Initially, survival analysis methods were used to investigate this association of PVD or UE score at PBE and other explanatory variables on time to pregnancy (DO) in the breeding season. In this analysis, time at risk was defined as EESD to pregnancy (i.e., the event) but for right censored data (i.e., where pregnancy was not observed) this was defined as the period from EESD to end of the mating period. Kaplan-Meier survival curves were used to visually assess and estimate median time to pregnancy dependent on PVD or UE Scores at the PBE and other explanatory variables. Log-log plots were also constructed to visually assess for time dependency assumptions prior to constructing the Cox proportional hazards models.

Explanatory categorical variables that were available in the dataset and that were initially tested with Kaplan-Meier and log-log plots, and included farm (8 levels); year (5 levels); parity (1 to ≥ 5); breed [coded as Holstein Friesian, (>11/16), Jersey (>11/16), or crossbred (>85% of which were Jersey cross)]; calving difficulty score (1, 2 & 3/4); twins (Yes, No); RFM (Yes, No); stillbirth (Yes, No); single calf sex (Male, Female); sire type (dairy or beef and AI or stock bull); BCS at calving (thin <3; ideal 3–3.25; fat ≥3.5), BCS at PBE (thin < 2.75; ideal 2.75–3.25; fat ≥3.5); the presence of a corpus luteum at PBE (Yes, No); PVD score (0 to 3); UE score (0 to 4) and time of PBE relative to EESD (Examined with ≥3 weeks to EESD; Examined with <3weeks to EESD).

Explanatory continuous variables in the dataset included DIM at EESD (i.e., DIM at entering the breeding season), predicted 305-day milk yield, genetic merit indices values for production (PTA for milk in kilograms; PTA for milk fat; PTA for milk protein) and fertility (PTA for calving interval and PTA for survival). Initially, the distribution of each continuous variable was visually assessed for normality using the 'ggplot' function and correlation tested using the Pearson correlation coefficient ('cor' function). When 2 variables were highly correlated (i.e., the coefficient was >0.8, e.g., in the case of PTA for milk in kilograms with PTA for milk protein and PTA for calving interval and PTA for survival), only 1 was selected (i.e., PTA for milk in kilograms and PTA for calving interval) and brought forward for the further analysis. These explanatory continuous variables were also divided into quartiles in order to initially test these as categorical variables also using Kaplan-Meier and log-log plots.

Initially, univariable Cox-proportional hazard models were built with DO (i.e, time to pregnancy) as the outcome to assess unconditional associations between each of the explanatory (categorical and continuous listed above) variables and the outcome. As there was clustering in the dataset (i.e., repeated measurements of individual cows), cow was included as a random effect in the univariable models. Additionally, DIM at EESD was forced into the univariable models to correct for DIM at entry into the study.The assumed proportional hazards were checked for by testing the survival time versus scaled Schoenfeld residuals using chi squared tests and visual appraisal of plots. The results of this univariable analysis of all these explanatory are included in the supplementary materials (Section 3.7, Table 3.8) for descriptive purposes.

3.3.9 Association between RTD diagnostic scores and subsequent reproductive outcome

The explanatory variables of primary interest in our study were either the PVD or UE Score at the time of PBE and the outcome of interest was the hazard of pregnancy within the subsequent breeding season. Based on this and prior to constructing the multivariable Cox hazards model, a directed acyclic graph was built after detailed discussions amongst the authors, and using freely available online resource DAGitty v. 3.0 software (Textor et al., 2016). This assisted in identifying any potential confounders (Figure 3.1; http://www.dagitty.net/dags.html?id=wgSiW1). Based on the directed acyclic graph, the minimal sufficient adjustment sets for estimating the total and direct effects of either PVD or UE Score on hazard of pregnancy within the breeding season were; parity, pre-breeding BCS, PTA for calving interval, the presence of a corpus luteum at PBE and the time of PBE relative to EESD. Variables that were dependent on herd were not included in the directed acyclic graph because cow-level variables that were generalisable to other farms were the focus of our present analysis.

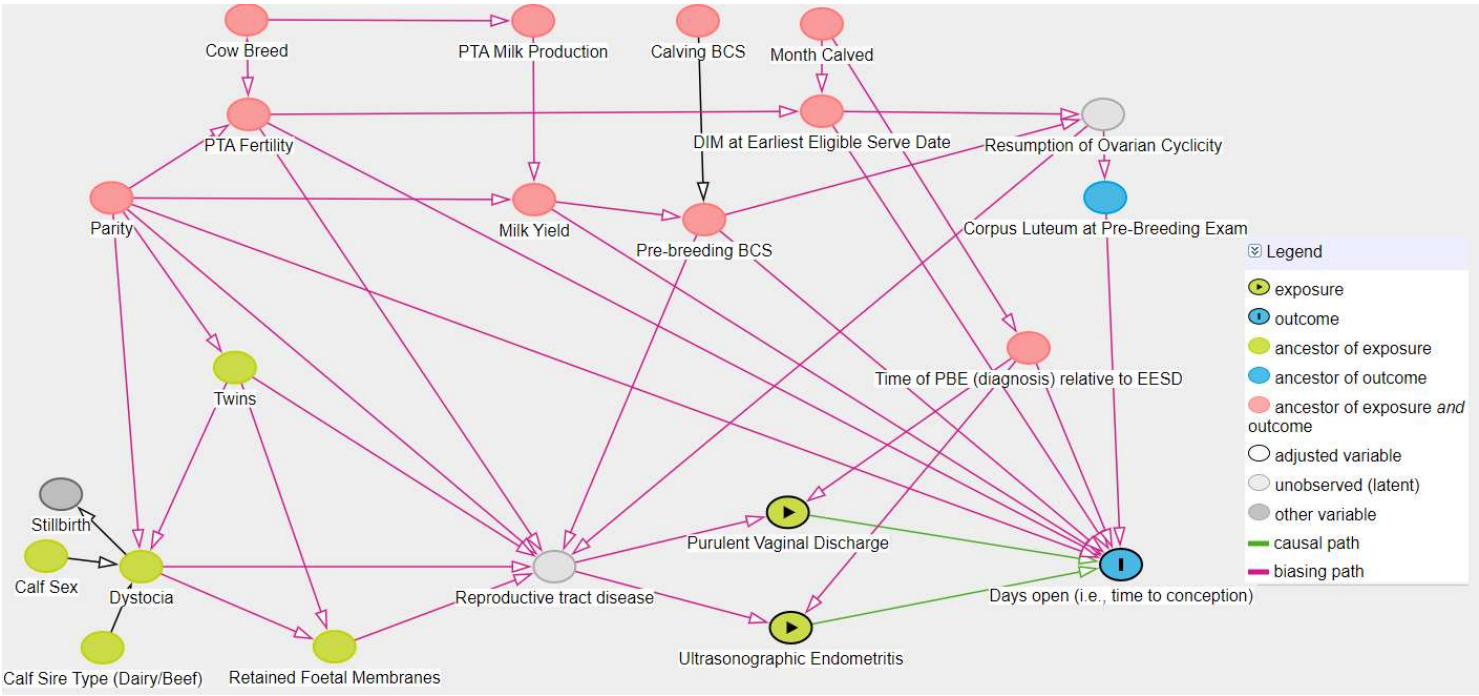

Figure 3.1 Directed acyclic graph used to inform the construction of the Cox Proportional Hazards Model to identify the association of purulent vaginal discharge (PVD) or ultrasonographic endometritis (UE) score at the pre-breeding examination (PBE) along with other potential explanatory variables on days open (i.e. time to conception) within the breeding season (available at; http://www.dagitty.net/dags.html?id=wgSiW1). The analysis was based on 4756 pre-breeding examinations performed on 2338 cows from 8 Irish seasonally calving pasture-based dairy herds over a 5-year period. The directed acyclic graph was constructed online with DAGitty version 3 (Textor et al., 2016).

Natural clustering occurred within the dataset, i.e., within herds and with repeated measurements of individual cows over multiple breeding seasons. To account for this clustering, the Cox model was stratified by herd while cow identification was included as a random effect. This allowed for the model to account for any unknown herd-level or cow-level variables. As in the univariable analysis, DIM at EESD was forced into the models to correct for DIM at entry into the study. Two separate Cox models were constructed to assess the individual impact of PVD or UE scores on the hazard of pregnancy within the subsequent breeding season. In the final multivariable models, each variable was evaluated using the likelihood ratio test ($P < 0.05$ were considered statistically significant) and the assumed proportional hazards were checked for by testing the survival time versus scaled Schoenfeld residuals using chi squared tests and visual appraisal of plots.

An interaction between time of PBE relative to EESD and PVD or UE Score was considered biologically relevant by the authors and forced into each of the multivariable models. The logic behind its' inclusion was that the further away from EESD that a cow was diagnosed the greater time and therefore chance of self or medical cure (i.e., in those that were treated as detailed above) prior to breeding than those diagnosed closer to or indeed after their EESD. In order to visualize this interaction in each models, we predicted the probabilities from each multivariable Cox analysis for the variables of interest (i.e., time of PBE relative to EESD and PVD or UE Score) while holding all other variables at the referent and mean of the population. Theses predicted probabilities are present in Figures 3.5 and 3.6 and assist in interpretation of the model outputs.

3.3.10 Bayesian Latent Class Analysis to determine RTD test sensitivity and specificity

Bayesian Latent class modelling was used to assess the Se and Sp of PVD or UE scoring to diagnose RTD at the time of the PBE and to estimate the within herd prevalence of RTD in each of the participating herds. The target condition in this analysis was RTD status of each cow (i.e., positive versus negative for RTD). This condition could include cows with cranial vaginitis, cervicitis, clinical and subclinical endometritis. Both tests are established cow side diagnostics to diagnose infectious and/or inflammatory conditions of the bovine reproductive tract and a positive status in either test has been shown in numerous studies to predict the probability of success in subsequent reproductive performance (Mee et al., 2009; McDougall et al., 2020). In terms of each of the test methods assessed, the test Se referred to cows with RTD and identified as such by a test while test Sp referred to cows without RTD and identified as such by a test. Figure 3.2 (overleaf) illustrates how the tests under evaluation relate to the target condition.

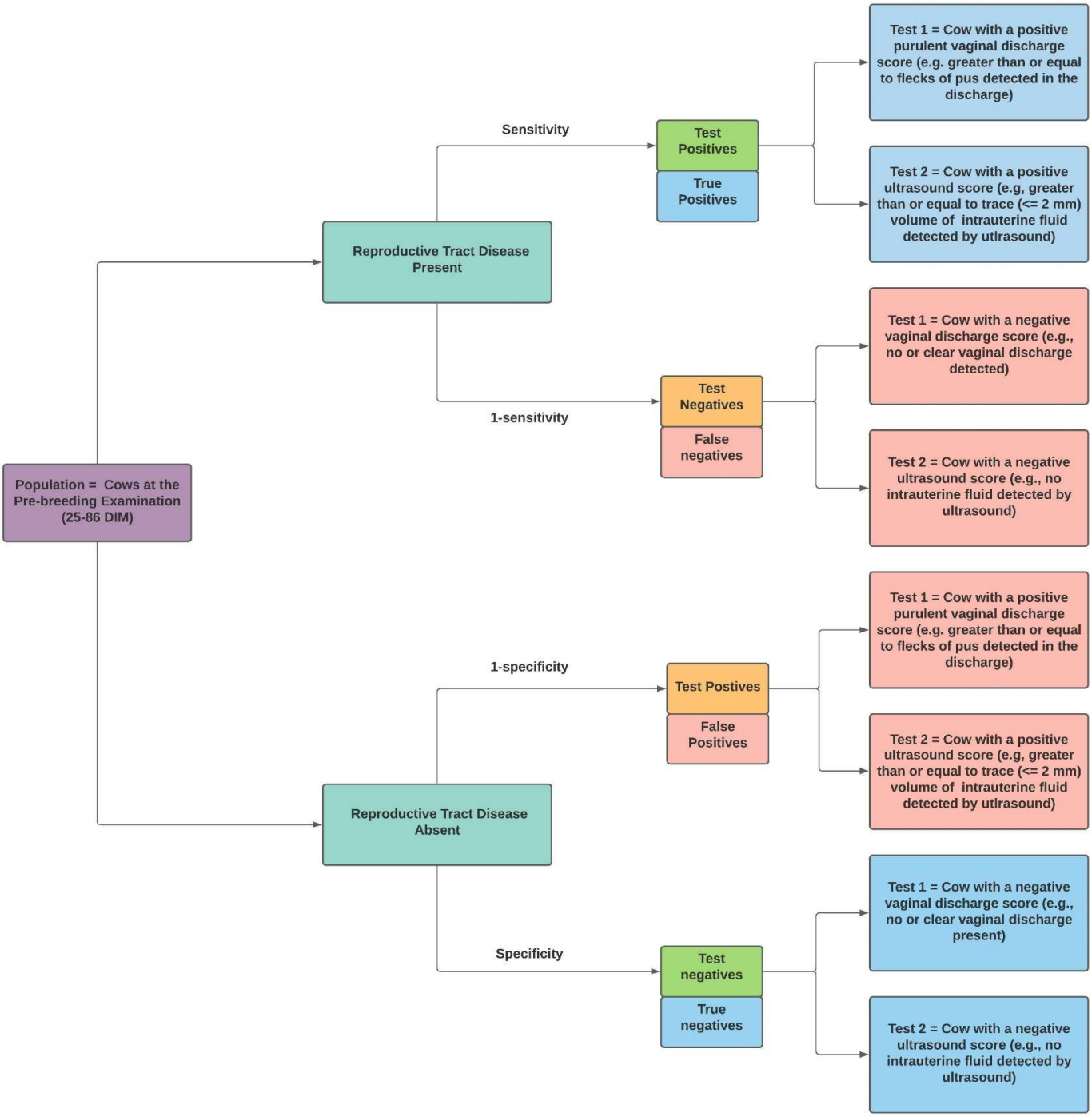

Figure 3.2 A flow diagram to illustrate how the tests under evaluation relate to the target condition (reproductive tract disease) used in the Bayesian latent class analysis.

As the PBEs in the dataset were clustered within cow and to avoid biased estimates from repeated sampling from the same cows in the dataset, only a single PBE was selected per cow to run the Bayesian latent class model. Consequently, cows with multiple PBEs in the dataset had a single PBE randomly selected from their available PBEs while those with only a single PBE were retained in the dataset. Thus, data used in this analysis was from 2460 PBEs in 2460 individual cows on the 8 farms.

The Bayesian latent class analysis used was similar in model structure to that proposed by Branscum et al. (2005), i.e., a two-tests multiple population model and the code used for model construction is provided in online supplementary materials (DOI; 10.17632/8xj77zy44r.1). Within herd prevalence and test Se and Sp test were modelled using prior beta distributions. Parameters of each beta distribution were estimated using an online free to use distribution calculator (Betabuster; https://shiny.vet.unimelb.edu.au/epi/beta.buster/). Informative priors for the Bayesian latent class model were chosen for within herd prevalence and test Se and Sp of PVD scoring based on previous published work (Arango-Sabogal et al., 2019, McDougall et al., 2020). Flat (i.e., non-informative) beta distributions (1, 1) were used for the Se and Sp of UE scoring considering that there is little published, using similar methodology, about its accuracy in determining RTD status at the time of sampling.

For within herd prevalence, a mode of 20%, and 5th percentile of 10% was chosen as this was the same proposed by Arango-Sabogal et al. (2019) and McDougall et al. (2020) and it was similar to the median posterior within herd prevalence estimate that McDougall et al. (2020) (22.9%; 95% BPI: 10.4-40.1%) found in their sample of seasonally calving pasture based dairy cows. The corresponding beta parameters for this distribution were 5.2 and 17.6.

The PVD Se prior estimate (mode = 65%, 5th percentile = 45%) and Sp prior estimate (mode = 90%, 5th percentile = 80%) were selected based on two recent publications using similar methodology. Arango-Sabogal et al. (2019), found a median (95% BPI) Se and Sp for PVD scoring of 64.2% (95% BPI; 59.1–70.3%) and 96.6% (95% BPI; 91.3–99.8%), respectively while McDougall et al. (2020), found a median Se and Sp for PVD scoring of 70.7% (95% BPI; 46.8–93.4%) and 88.1% (95% BPI; 81.3–95.4%), respectively. The corresponding beta parameters for PVD scoring Se and Sp were (12.2, 7.0) and (42.6, 5.6).

The model assumed conditional dependence between tests. This assumption was based on the fact that although both tests are examining different parts of the reproductive tract (i.e., PVD scoring is assessing character of cranial vaginal discharge and UE scoring is assessing appearance and volume of intrauterine fluid), both tests are attempting to visually quantify inflammatory material produced in the reproductive tract primarily as the result of infection. This means that a greater degree of infection and inflammation should cause the sensitivity of both tests to increase, setting up a positive dependence between the tests among cows suffering from RTD. Additionally, correlation between the tests might indeed result from

unmeasured factors in our sample and ignoring conditional dependence has the potential to bias estimates (Wang et al., 2017).

The Bayesian latent class model structure is outlined as follows. For the k-th herd, the nk observations give the data vector for the joint test results y11, y10, y01, y00. Where y11 is the number of animals from the kth herd that test positive on PVD and UE score and y10 is the number that test positive on PVD and negative on UE score and so on. y1 to yk are assumed to have multinomial sampling distributions:

$y_k \sim \text{multinomial}(n_k, (p_{11k}, p_{10k}, p_{01k}, p_{00k}))$

The multinomial cell probabilities are given by:

$p_{11k} = \pi_k \times (Se_{PVD} \times Se_{UE} + Cov_{SE}) + (1 - \pi_k) \times ((1 - Sp_{PVD}) \times (1-Sp_{UE}) + Cov_{SP})$

$p_{10k} = \pi_k \times (Se_{PVD} \times (1-Se_{UE}) - Cov_{SE}) + (1 - \pi_k) \times ((1 - Sp_{PVD}) \times Sp_{UE} - Cov_{SP})$

$p_{01k} = \pi_k \times ((1-Se_{PVD}) \times Se_{UE} - Cov_{SE}) + (1 - \pi_k) \times (Sp_{PVD} \times (1-Sp_{UE}) - Cov_{SP})$

$p_{00k} = \pi_k \times ((1-Se_{PVD}) \times (1-Se_{UE}) + Cov_{SE}) + (1 - \pi_k) \times (Sp_{PVD} \times Sp_{UE} - Cov_{SP})$

Where π_k is the prevalence in the k-th herd and Se_{PVD}, Se_{UE}, Sp_{PVD} and Sp_{UE} are the sensitivities and specificities of PVD and UE respectively, and Cov_{SE} and Cov_{SP} represented the pairwise covariance between the two tests for diseased and non-diseased animals respectively.

Posterior inferences for test Se, Sp, and the within prevalence for each herd were obtained using MCMC sampling using JAGS (Plummer, 2003), run using the 'rjags' package (Plummer et al., 2019) in R studio (R Core Team, 2015). Initial trial runs demonstrated evidence of chain autocorrelation [i.e., low effective sample size values (SSeff) and autocorrelation plots]; therefore, after a burn-in period of 5,000 iterations, models were run for total of 150,000 iterations with chains thinned to every 10^{th} iteration from an inference sample of 15,000. Convergence of the Markov chains was assessed by visual assessment of Markov chain and autocorrelation plots and by running multiple (n = 2) chains from distinct starting values (e.g., 0.05 and 0.95 for variables bounded between 0 and 1). Optimum thresholds for each test were defined using the misclassification cost terms (**MCT**). The MCT was calculated as per Grenier (1996) with the following equation.

$MCT = (1-p)*(1-Sp) + (r*p)*(1-Se)$

Where p is the disease prevalence estimate and r is the false negative to false positive cost ratio. A fixed value of p was set as 0.2 as the median for the final model within herd prevalence. Similar to the methodology of Arango-Sabogal et al. (2019), a range of false negative to false positive ratios of 1:1, 1:3 and 1:5 were assessed in the above equation. The rational argued by these authors is that a false negative to false positive ratio of 1:1 considers that on treating a false positive with an antimicrobial is

at least as problematic as not treating a false negative while a ratio of 1:5 considers that on treating a false positive with an antimicrobial is much more problematic than missing a false negative. Additionally, a false negative to false positive ratio of 2:1 was considered and assessed as some authors (de Boer et al., 2014) have argued that the cost of an untreated case of RTD (false negative) is a greater economic cost to seasonal dairy farmers than treating a number of false positive cases of RTD with an intrauterine antibiotic such as, cephapirin. The reasoning that is argued is that in seasonal herds diagnosis is often only carried out on one occasion prior to the breeding season and that time to intervene is limited compared to that of year-round calving systems. (de Boer et al., 2014).

The MCT was calculated using each possible threshold for each of the two diagnostic tests evaluated (i.e., PVD or UE scoring). For PVD scoring threshold determination, 8 models were run varying the thresholds from 0 to 3 and using fixed thresholds for UE (i.e.,≥ 2: small amount of intraluminal fluid or ≥ 1: trace amount of intraluminal fluid). These fixed thresholds for UE score are what has been considered positive for disease and shown to impact on reproductive outcome in our current dataset (i.e., UE score ≥1) and in other research (i.e., UE score ≥2; Mee et al., 2009). For UE scoring threshold determination, 10 models were run by varying the thresholds from score 0 to score 4 and using a fixed threshold for PVD (i.e., ≥ 2: mucopurulent discharge or ≥ 1: flecks of pus). These fixed thresholds for PVD score are what are has been considered positive for disease and shown to impact on reproductive outcome in our current dataset (i.e., PVD score ≥2) and in other research (i.e., PVD score ≥1; Giuliodori et al., 2017). Once an optimal threshold was chosen for each diagnostic test, positive predictive values and negative predictive values were calculated for each test on each farm based on the median estimated within herd prevalence from the final model.

As part of our sensitivity analysis, several measures were undertaken including relaxing the prior assumptions for PVD scoring and sub-setting the dataset based on DIM at PBE. Firstly, to assess the prior priors assumptions for PVD scoring the Se and Sp were given flat prior distribution [β (1, 1)]. Secondly, the dataset was subdivided into two subsets based on DIM at PBE (25-45 DIM and 46-86 DIM) to examine if there was constant accuracy of diagnostic tests over time when sampled.

3.4 Results

3.4.1 Descriptive Statistics

A total 5,049 PBEs on 2,460 spring-calving cows from 8 farms were conducted each spring over a 5-year period. The median DIM at PBE was 49 DIM (interquartile range = 21 d). There was a median of 2 PBEs per cow (range 1-5) within the dataset. Complete reproductive data was available on 4756 PBEs on a total of 2338 cows on the 8 farms over the 5-year period and were used in the reproductive performance analysis. A total of 2460 PBEs conducted on 2460 individual cows was used in the Bayesian latent class model to determine test Se and Sp.

All routine seasonal reproductive performance outcomes for each PBE test classification and in the overall dataset (n=4756) are summarized in Table 3.2. For the complete set of PBEs analyzed, the mean (95% CI) proportion of cows submitted within the first 24 days of their EESD was 88% (95% CI; 87-89%), the mean (95% CI) pregnancy per AI to the first AI was 56% (95% CI; 54-57%), the mean (95% CI) DO was 34.5 (95% CI; 33.6-35.3) days and the mean (95% CI) proportion of pregnant cows within the first 6 weeks after MSD was 69% (95% CI; 68-70%).

Table 3.2 also implies that most reproductive performance outcomes were negatively impacted as a function of increasing score of each PBE test result (i.e., PVD or UE scores). The results of the Kaplan–Meier curves for both PVD and UE scores are displayed in Figures 3.3 and 3.4, respectively. Vertical dotted lines from each score on both curves indicate the median DO for each score. Again, these curves imply that DO increased as a function of increasing score on each PBE test. However, it must be emphasized that both data presented in Table 3.2 and in the Kaplan–Meier curves are from univariable and unadjusted analyses that is provided for descriptive purposes only. The results of the initial univariable Cox-proportional hazard models are available in the supplementary materials (Section 3.7, Table 3.8).

Table 3.2. Descriptive summary of the of reproductive performance outcomes associated with purulent vaginal discharge (PVD) score or ultrasonographic endometritis (UE) score category from 4756 pre-breeding examinations conducted on 2338 cows in 8 Irish seasonally calving pasture-based dairy herds over the 5-year period.

Pre-breeding Examination Test	Class	n	Mean Proportion of cows submitted for AI within 24 days after EESD (95%CI)	Mean First Serve Pregnancy per AI (95%CI)	Mean Days Open with breeding season (95%CI)	Mean Proportion of cows pregnant 6 weeks after MSD (95%CI)
PVD score	0	3959	0.89 (0.88-0.90)	0.57 (0.56-0.59)	33.4 (32.5-34.3)	0.70 (0.69-0.72)
	1	448	0.87 (0.85-0.91)	0.52 (0.47-0.57)	36.3 (33.4-39.2)	0.67 (0.63-0.72)
	2	184	0.80 (0.74-0.86)	0.50 (0.42-0.57)	41.3 (36.5-46.0)	0.59 (0.52-0.66)
	3	165	0.81 (0.76-0.88)	0.41 (0.34-0.49)	46.7 (41.5-51.9)	0.55 (0.47-0.62)
UE score	0	3753	0.90 (0.89-0.91)	0.58 (0.56-0.60)	32.6 (31.7-33.5)	0.71 (0.70-0.73)
	1	685	0.86 (0.84-0.89)	0.52 (0.48-0.56)	38.0 (35.6-40.3)	0.65 (0.61-0.69)
	2	158	0.77 (0.71-0.84)	0.41 (0.33-0.49)	45.1 (39.8-50.3)	0.56 (0.48-0.64)
	3	89	0.79 (0.70-0.87)	0.45 (0.34-0.56)	44.7 (38.0-51.5)	0.56 (0.46-0.67)
	4	71	0.68 (0.56-0.79)	0.22 (0.12-0.33)	61.8 (54.0-69.6)	0.34 (0.23-0.45)
Overall		4756	0.88 (0.87-0.89)	0.56 (0.54-0.57)	34.5 (33.6-35.3)	0.69 (0.68-0.70)

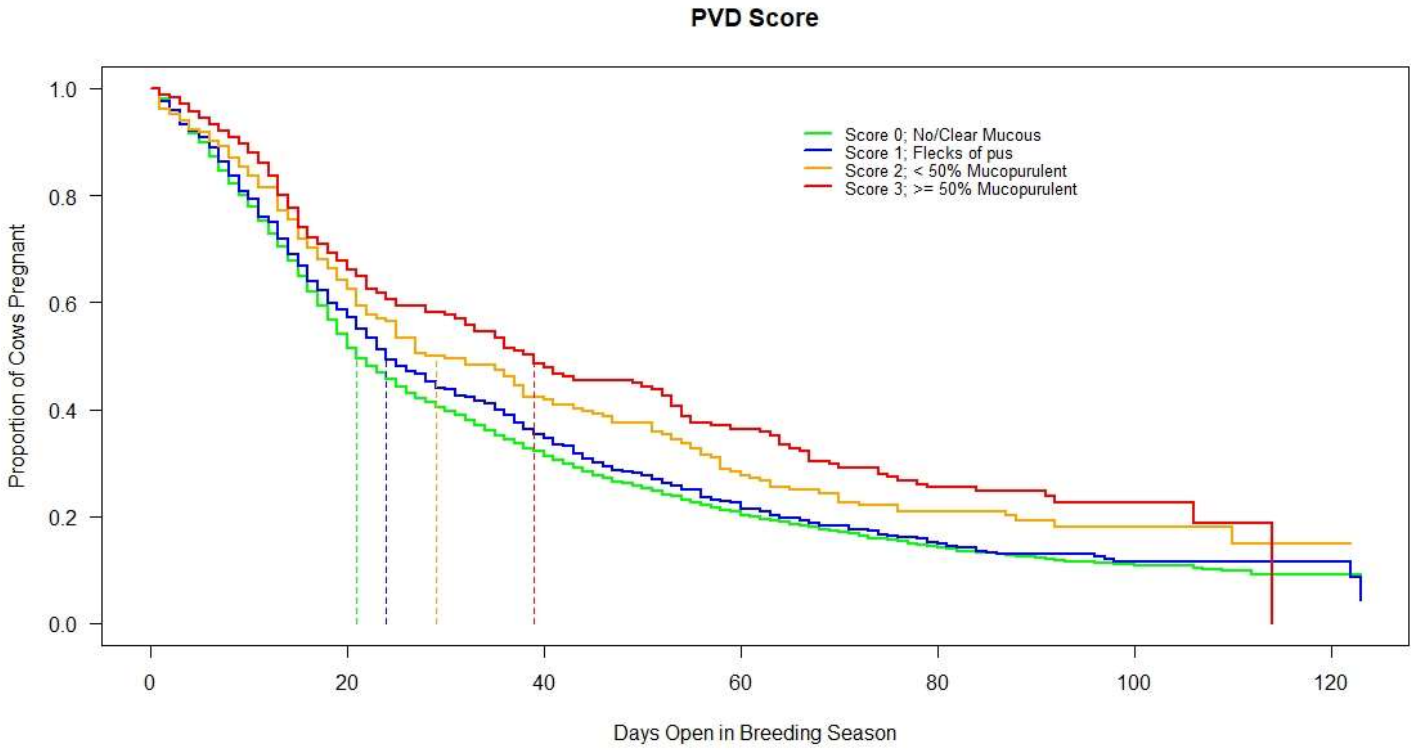

Figure 3.3 Kaplan–Meier survival curve illustrating the association between purulent vaginal discharge (PVD) score category and days open (i.e., time to conception) within the breeding season. The analysis was based on 4756 pre-breeding examinations performed on 2338 cows from 8 Irish seasonally calving pasture-based dairy herds over a 5-year period. In this curve, the median survival time is indicated by a colored vertical dashed line corresponding to each category. Those pre-breeding examinations that showed no evidence of reproductive tract disease diagnosed by Metricheck (score 0) had lowest median days open and the steepest survival time (i.e., conceived the fastest).

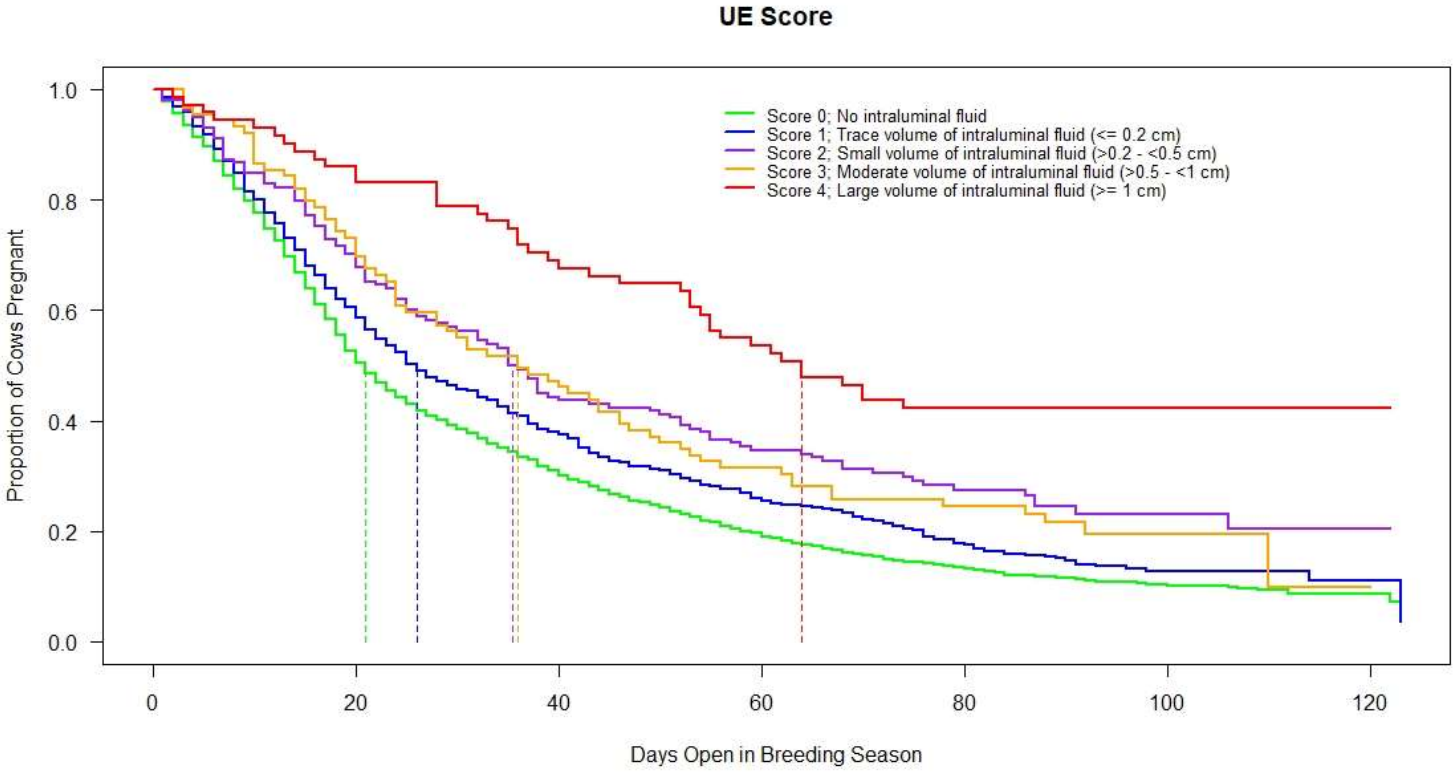

Figure 3.4 Kaplan–Meier survival curve illustrating the association between ultrasonographic endometritis (UE) score and days open (i.e., time to conception) within the breeding season. The analysis was based on 4756 pre-breeding examinations performed on 2338 cows from 8 Irish seasonally calving pasture-based dairy herds over the 5-year period. In this curve, the median survival time is indicated by a colored vertical dashed line corresponding to each category. Those pre-breeding examinations that showed no evidence of reproductive tract disease (score 0) diagnosed by ultrasound had lowest median days open and the steepest survival time (i.e., conceived the fastest).

3.4.2 Association between RTD diagnostic scores and subsequent reproductive outcome

The outputs from the final multivariable Cox proportional hazards model examining the association of PVD or UE score at PBEs on the hazard of pregnancy within a breeding season are illustrated in Tables 3.3 and 3.4, respectively . Cows with PVD score of 2 or 3 at the PBE were less likely to conceive than cows with PVD score of 0 (score 2 HR = 0.74; 95% CI: 0.59-0.94; score 3 HR = 0.65; 95% CI: 0.51-0.84). Cows with UE score of 1, 2, 3 or 4 were less likely to conceive than cows with UE score of 0 (score 1 HR = 0.82; 95% CI: 0.73-0.93; score 2 HR = 0.79; 95% CI: 0.62-1.00; score 3 HR = 0.62; 95% CI: 0.43-0.90; score 4 HR = 0.39; 95% CI: 0.26-0.58). As the interaction between test scores and time of the PBE relative to the EESD term, were forced into both models, the HR results presented above of either test scores should not be interpreted in isolation of this interaction.

The plots of the predicted probabilities generated from each model graphically illustrate this interaction well and are presented in Figures 3.5 and 3.6. The predicted plots from the PVD model illustrate that if a PBE is conducted ≥ 3 weeks before EESD, then PVD scores of 2 or 3 appear to have impaired predicted subsequent reproductive performance whereas score 0 or 1 were remarkably similar. However, if the if the PBE is conducted within the 3 weeks before EESD, then PVD scores of 1 or 2 or 3 all appear to have impaired predicted subsequent reproductive performance versus score 0. Also of note is the degree of impact each score has on subsequent reproductive performance, with PVD lower scores that are diagnosed closer to the breeding period (EESD) having a greater association with impaired reproductive performance. PVD score 1 was not found to be associated with impaired reproductive performance in the present model but based on the predicted probability plots, there was a tendency to be associated with impaired performance when a cow is examined closer to the time of breeding.

The predicted plots from the UE model illustrates that UE scores of 1 or 2 or 3 or 4, have impaired predicted subsequent reproductive performance compared to score 0 in both time period categories relative to EESD that were assessed. Like in the PVD model, the degree of impact the degree of impact each score has on subsequent reproductive performance tended to be greater for lower scores when they that are diagnosed closer to the breeding period (i.e., a cow's EESD).

Other notable variables that were associated with hazard of pregnancy within the breeding season and included in the final Cox model were; parity, BCS at the PBE, PTA for calving interval (genetic index), DIM at EESD, and these results are also presented in Tables 3.3 and 3.4. In both the PVD and UE models, multiparous cows were less likely to conceive than first parity cows (e.g., for the PVD model; 3rd parity HR = 0.84; 95% CI: 0.77-0.92; 4th parity HR = 0.80; 95% CI: 0.72-0.89; ≥5th Parity HR = 0.65; 95% CI: 0.59-0.72). In both the PVD and UE models, thin cows (i.e., <2.75 out of 5 BCS) at the PBE, had a reduced hazard of subsequent pregnancy (HR = 0.86; 95% CI 0.75-0.99). DIM at EESD which is a continuous variable and a measure of the DIM when a cow enters the breeding season and is a product of her previous calving date, was associated with hazard of pregnancy within the breeding

season (HR = 1.012; 95% CI 1.010-1.014). This association indicates that as DIM at EESD increased (i.e., the earlier in the calving season a cow calved and thus the more DIM at MSD/EESD) the greater the likelihood of pregnancy. PTA for calving interval which is a continuous variable that is a genetic prediction of how long the calving interval will be for an individual cow was associated with the hazard of pregnancy within the breeding season (HR = 0.92; 95% CI 0.90-0.93). This association indicates that as PTA for calving interval value increases (i.e. the more likely the cow is to have a long calving interval or negative predicted reproductive performance) the lower the hazard of pregnancy within the breeding season.

Table 3.3. Multivariable Cox proportional hazards model assessing the associations between purulent vaginal discharge score (PVD) score at pre-breeding examination (PBE) on the hazard of pregnancy within a breeding season. A total of 4756 PBEs were analyzed on 2338 cows in 8 Irish seasonally calving pasture-based dairy herds over the 5-year period. The analysis was stratified by herd, and cow was a included as a random effect in the model. Additional variables included in the model were; the time of PBE relative to earliest eligible service date (EESD), parity, pre-breeding BCS, the presence of a corpus luteum at PBE, permitted transmitting ability (PTA) for calving interval, DIM at EESD and the interaction between PVD score and the time of PBE relative to earliest eligible service date (EESD).

Variable	Class	n	Coefficient	Hazard Ratio	95% CI	*P*-value
PVD Score	0	3959		Referent		
	1	448	-0.12	0.88	0.75-1.04	0.14
	2	184	-0.30	0.74	0.59-0.94	0.01
	3	165	-0.43	0.65	0.51-0.84	<0.01
Time of PBE relative to EESD	<3 weeks to EESD	2967		Referent		
	≥3 weeks to EESD	1789	0.02	1.02	0.94-1.10	0.70
Parity	1	1476		Referent		
	2	1151	-0.05	0.95	0.87-1.03	0.20
	3	823	-0.17	0.84	0.77-0.92	<0.01
	4	569	-0.22	0.80	0.72-0.89	<0.01
	5+	737	-0.43	0.65	0.59-0.72	<0.01
PBE BCS	2.75-3.25	4363		Referent		
	≥3.5	66	0.04	1.04	0.80-1.34	0.77
	<2.75	327	-0.15	0.86	0.75-0.99	0.03
CL Present at PBE	Yes	3491		Referent		
	No	1265	-0.07	0.93	0.87-1.00	0.06
PTA Calving Interval	-	4756	-0.09	0.92	0.90-0.93	<0.01
DIM at EESD	-	4756	0.01	1.012	1.010-1.014	<0.01
Interactions	Score 1 × ≥3 weeks to EESD		0.12	1.12	0.91-1.39	0.29
	Score 2 × ≥3 weeks to EESD		0.22	1.25	0.90-1.74	0.19
	Score 3 × ≥3 weeks to EESD		0.12	1.12	0.79-1.60	0.52

Table 3.4. Multivariable Cox proportional hazards model assessing the associations between ultrasonographic endometritis (UE) score at pre-breeding examination (PBE) on the hazard of pregnancy within a breeding season. A total of 4756 PBEs were analyzed on 2338 cows in 8 Irish seasonally calving pasture-based dairy herds over the 5-year period. The analysis was stratified by herd, and cow was a included as a random effect in the model. Additional variables included in the model were; the time of PBE relative to earliest eligible service date (EESD), parity, pre-breeding BCS, the presence of a corpus luteum at PBE, permitted transmitting ability (PTA) for calving interval, DIM at EESD and the interaction between UE score and the time of PBE relative to earliest eligible service date (EESD).

Variable	Class	n	Coefficient	Hazard Ratio	95% CI	*P*-value
UE Score	0	3753		Referent		
	1	663	-0.20	0.82	0.73-0.93	<0.01
	2	144	-0.24	0.79	0.62-1.00	0.05
	3	84	-0.48	0.62	0.43-0.90	0.01
	4	71	-0.98	0.39	0.26-0.58	<0.01
Time of PBE to EESD	<3 weeks to EESD	2967				
	≥3 weeks to EESD	1789	0.02	1.02	0.94-1.11	0.62
Parity	1	1476		Referent		
	2	1151	-0.05	0.95	0.88-1.03	0.24
	3	823	-0.16	0.85	0.78-0.93	<0.01
	4	569	-0.20	0.82	0.74-0.91	<0.01
	5+	737	-0.41	0.66	0.60-0.74	<0.01
PBE BCS	2.75-3.25	4363		Referent		
	≥3.5	66	-0.01	0.99	0.77-1.28	0.97
	<2.75	327	-0.16	0.86	0.75-0.98	0.03
CL Present at PBE	Yes	3491		Referent		
	No	1265	-0.06	0.94	0.87-1.01	0.09
PTA Calving Interval		4756	-0.08	0.92	0.90-0.93	<0.01
DIM at EESD		4756	0.012	1.012	1.010-1.014	<0.01
Interactions	Score 1 × ≥3 weeks to EESD		0.14	1.15	0.96-1.37	0.13
	Score 2 × ≥3 weeks to EESD		-0.18	0.84	0.58-1.22	0.36
	Score 3 × ≥3 weeks to EESD		0.41	1.51	0.93-2.45	0.09
	Score 4 × ≥3 weeks to EESD		0.13	1.14	0.61-2.14	0.68

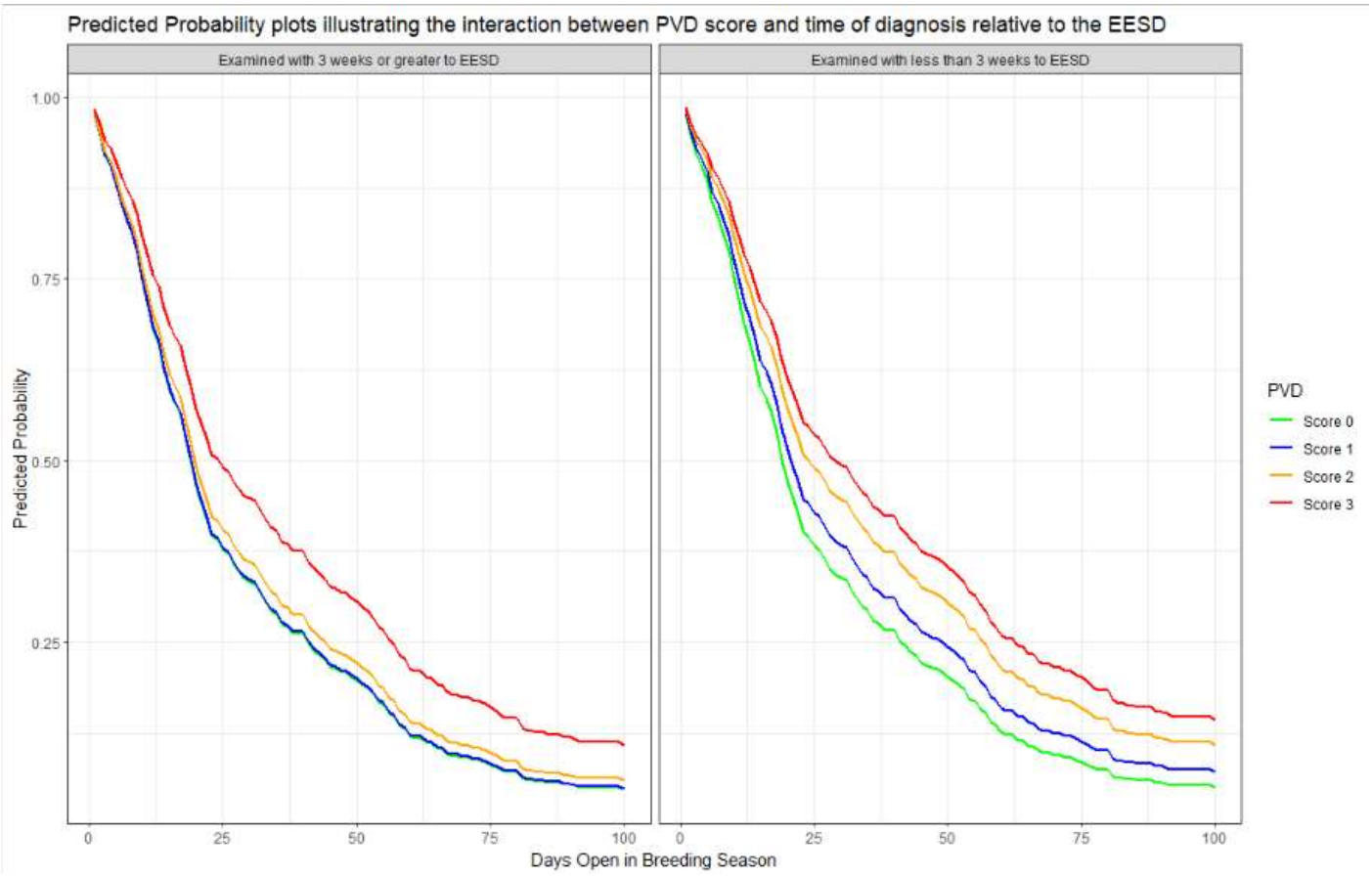

Figure 3.5 Predicting probabilities the from the multivariable Cox model. The plot illustrates the interaction between the time between diagnosis and the earliest eligible service date (EESD) and the relative purulent vaginal discharge (PVD) score and its'association with the probability of pregnancy in the breeding season. All the other covariates in the model were held the at the referent or mean of the population.

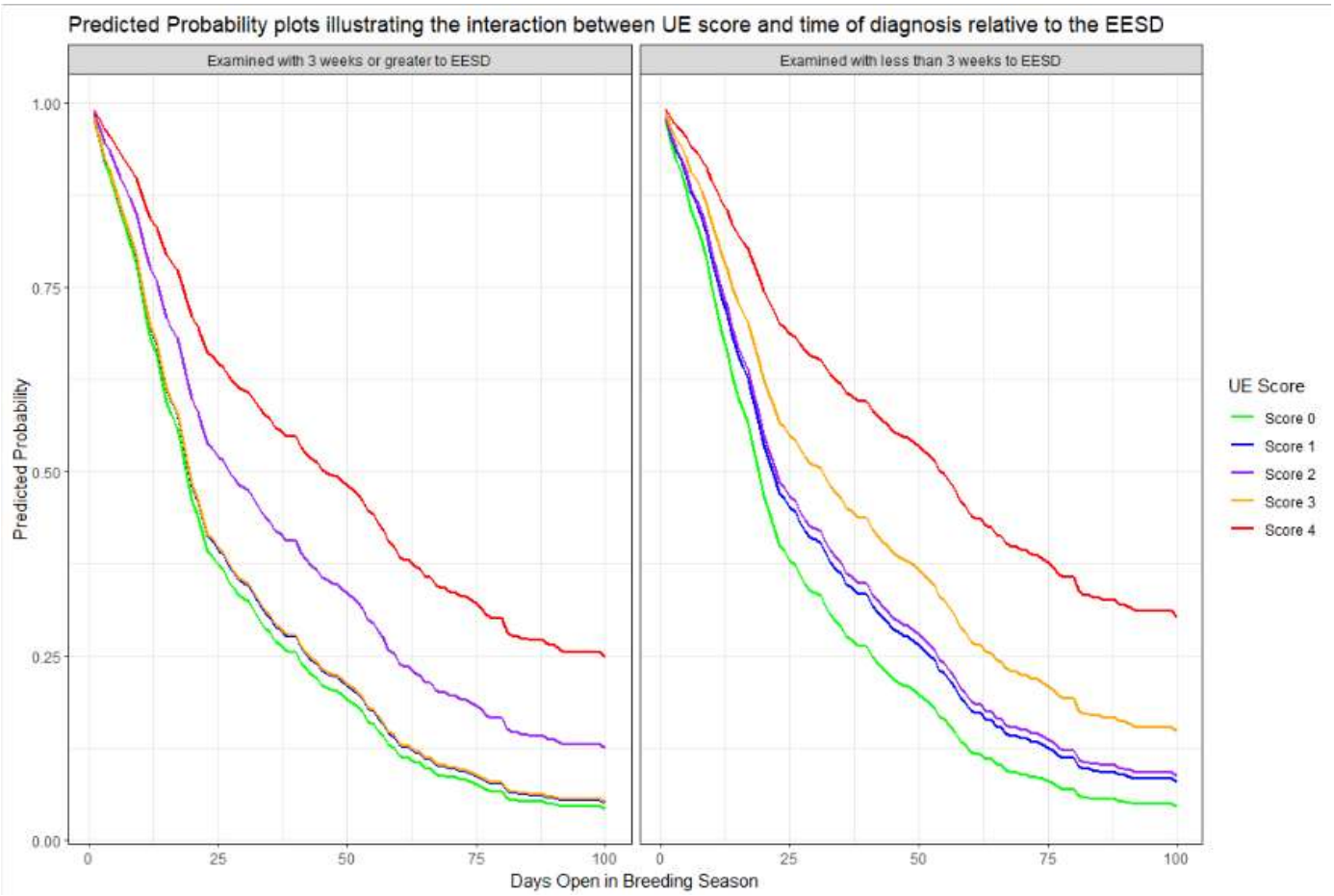

Figure 3.6 Predicting probabilities the from the multivariable Cox model. The plot illustrates the interaction between the time between diagnosis and the earliest eligible service date (EESD) and the relative ultrasonographic endometritis (UE) score and its' association with the probability of pregnancy in the breeding season. All the other covariates in the model were held the at the referent or mean of the population.

3.4.3 Bayesian Latent Class Analysis

Cross-tabulated test result used for the final Bayesian latent class model are presented as supplementary materials (Section 3.7, Table 3.9). The median posterior estimates for Se, Sp and their respective 95% Bayesian probability interval (**95% BPI**) for each of the diagnostic tests at the various thresholds (PVD scores 0 to 3; UE scores 0 to 4), when the other diagnostic test scores were fixed, are illustrated in Table 3.5. When the opposing diagnostic test threshold were fixed, score 1 for both PVD and UE showed the highest median Se estimates values [median PVD ≥ 1 Se = 44% (95% BPI: 28-62%) and 44% (95% BPI: 29-60%); UE ≥ 1 Se = 47% (95% BPI: 19-84%) and 67% (95% BPI: 34-100%)] while Sp remained relatively high for both [median PVD ≥ 1 Sp = 90% (95% BPI: 86-94%) and 90% (95% BPI: 86-93%); UE ≥ 1 Sp = 86% (95% BPI: 79-93%) and 91% (95% BPI: 84-100%)].

MCT was calculated at four different false negative to false positive ratios, using a static prevalence of RTD of 20% and the median posterior estimate for Se and Sp at the various thresholds and these results are visible in Table 3.5. The threshold for which the MCT values were absolute lowest varied depending on the false negative to false positive ratio and the test threshold selected. Figure 3.7 and 3.8 illustrate changes in MCT according to the changes in the test threshold. For both tests, MCT followed a similar pattern for 1:3 and 1:5 ratios, and a slightly different pattern for 2:1 and 1:1 ratio. For PVD scoring, the optimal threshold was at PVD score ≥ 1 for 2:1, PVD score ≥ 2 for the 1:1 ratio and at PVD score ≥ 3 for the 1:3 and 1:5 ratio. For UE scoring, the optimal threshold was at score ≥ 1 for the 2:1 and 1:1 ratio and at UE score ≥ 3 for the 1:3 and 1:5 ratio. However, based on the overall pattern of the changes to MCT with varying thresholds, and the marginal differences after increasing from cut-off points, we suggest that a cut-off of score ≥ 1 for either tests could be used as a consistent threshold to reflect adequate MCT, irrespective of the ratio used, and beyond which any improvements in MCT were likely to be marginal.

Based on the test Se and Sp estimates and the MCT assessment, our suggested optimal threshold for PVD or UE were fixed at scores of ≥ 1 and the complete results of the final model are illustrated in Table 3.6. The herds with the lowest and highest median (95% BPI) within herd prevalence estimates were herd 1 [14% (7-22%)] and herd 5 [27% (11-31%)], respectively. The positive predictive value for PVD score ≥ 1 in these same herds was 41% (14-70%) in herd 1 and 61% (31-84%) in herd 5 while the negative predictive value ranged from 91% (89-94%) in herd 1 and 81% (79-85%) in herd 5. Likewise in these herds, the positive predictive value for UE score ≥1 was 56% (13-100%) in herd 1 and 74% (29-100%) in herd 5 while the negative predictive value ranged from 94% (94-100%) in herd 1 and 88% (86-100%) in herd 5.

The complete results of the sensitivity analysis are illustrated in Table 3.7. Results of the model using flat priors for PVD for Se and Sp suggest that prior information may have impacted on the Se estimates of both in the final model. Median (95% BPI) Se estimates for PVD score ≥ 1 did alter between models

with 24% (95% BPI: 2-45%) versus 44% (95% BPI: 29-60%) in the non-informative and informative models, respectively. Median (95% BPI) Se estimates for UE score ≥ 1 also differed between models with 47% (95% BPI: 0-81%) versus 67% (95% BPI: 33-100%) noted in the non-informative and informative models, respectively. There was less of a difference in median (95% BPI) Sp estimates for PVD score ≥ 1 between models with 85% (95% BP: 79-91%) versus 90% (95% BPI: 86-93%) in the non-informative and informative models, respectively. Likewise, a similar difference was in median (95% BPI) Sp estimates for UE score ≥ 1 between the non-informative [88% (95% BPI: 76-98%)] and informative [91% (95% BPI: 83-100%)] models, respectively. However, it must be noted that the lack of direction that flat (i.e., non-informative) priors bring to Bayesian Latent class analysis can in of themselves bias the analysis and therefore some authors have cautioned about their use and interpretation (Lele, 2020).

The subdivision of the dataset into two categories based on DIM at examination (25-45 DIM and 46-86 DIM) was conducted to investigate if there was constant accuracy of diagnostic tests over the time period sampled and the results are presented in Table 3.7. This part of the sensitivity analysis illustrated a marked difference in estimates. In the case of PVD score, the Se median (95% BPI) estimate was 59% (95% BPI: 45-74%) when PBE took place between 25-45 DIM versus 44% (95% BPI: 29-61%) when the examination took place between 46-86 DIM and the Sp decreased from 94% (95% BPI: 91-97%) at the later PBE to 87% (95% BPI: 82-91%) at the earlier PBE date range. In the case of UE score, the difference in the median Se estimate was starker as it was 87% (95% BPI: 61-100%) when PBE took place between 25-45 DIM versus 36% (95% BPI: 7-71%) when the examination took place between 46-86 DIM. However, the median Sp estimate for UE score remained largely stable between both models, 90% (95% BPI: 82-97%) at the early DIM examination period versus 89% (95% BPI: 81-97%) at the later DIM period.

Table 3.5 Sensitivity (Se) and specificity (Sp) of ultrasonographic endometritis (UE) score and purulent vaginal discharge score (PVD) at different thresholds obtained using a Bayesian latent class analysis for the detection of reproductive tract disease at the pre-breeding examination in Irish dairy cows (n=2460) in 8 herds between 25-86 days in milk over a 5 year period. The model assumed conditional dependency between tests. A 95% Bayesian probability interval (BPI) is calculated for each Se and Sp estimate. A misclassification cost term (MCT) is calculated for each threshold based on median test Se and Sp.

Test	Threshold	Se (Median)	95% BPI	Sp (Median)	95% BPI	MCT [e] r=2:1	MCT [e] r=1:1	MCT [e] r=1:3	MCT [e] r=1:5
PVD [a]	Score 0	0.99	0.98-1.00	0.02	0.02-0.03	0.79	0.79	0.78	0.78
	Score 1	0.44	0.28-0.62	0.90	0.86-0.94	0.30	0.19	0.12	0.10
	Score 2	0.33	0.22-0.47	0.97	0.95-0.99	0.29	0.16	0.07	0.05
	Score 3	0.18	0.10-0.26	0.99	0.97-0.99	0.34	0.17	0.06	0.04
PVD [b]	Score 0	0.99	0.99-1.00	0.02	0.02-0.03	0.79	0.79	0.78	0.78
	Score 1	0.44	0.29-0.60	0.90	0.86-0.93	0.30	0.19	0.12	0.10
	Score 2	0.32	0.20-0.45	0.97	0.95-0.99	0.30	0.17	0.08	0.06
	Score 3	0.17	0.10-0.25	0.98	0.97-0.99	0.35	0.18	0.07	0.05
UE [c]	Score 0	1.00	0.99-1.00	0.00	0.00-0.00	0.80	0.80	0.80	0.80
	Score 1	0.47	0.19-0.84	0.86	0.79-0.93	0.32	0.22	0.15	0.13
	Score 2	0.24	0.10-0.40	0.96	0.94-1.00	0.34	0.18	0.08	0.06
	Score 3	0.15	0.06-0.26	0.99	0.97-1.00	0.35	0.18	0.06	0.04
	Score 4	0.07	0.01-0.13	0.99	0.98-1.00	0.38	0.19	0.07	0.05
UE [d]	Score 0	1.00	0.99-1.00	0.00	0.00-0.00	0.80	0.80	0.80	0.80
	Score 1	0.67	0.34-1.00	0.91	0.84-1.00	0.20	0.14	0.09	0.09
	Score 2	0.26	0.10-0.43	0.98	0.94-1.00	0.31	0.16	0.07	0.05
	Score 3	0.16	0.08-0.25	0.99	0.98-1.00	0.34	0.18	0.06	0.04
	Score 4	0.07	0.02-0.12	0.99	0.99-1.00	0.38	0.19	0.07	0.05

Table 3.5 Footnotes

[a] = UE score fixed at ≥ score 2 (small (>2 to <5 mm) volume intraluminal fluid)

[b] = UE score fixed at ≥ score 1 (trace (≤ 2 mm) volume intraluminal fluid)

[c] = PVD score fixed at ≥ score 2 (Mucopurulent)

[d] = PVD score fixed at ≥ score 1 (Flecks of pus)

[e] = MCT = $(1-p)*(1-Sp) + (r*p)*(1-Se)$, where p is prevalence and fixed at 0.2 and r is the false either 2:1, 1:1, 1:3 or 1:5

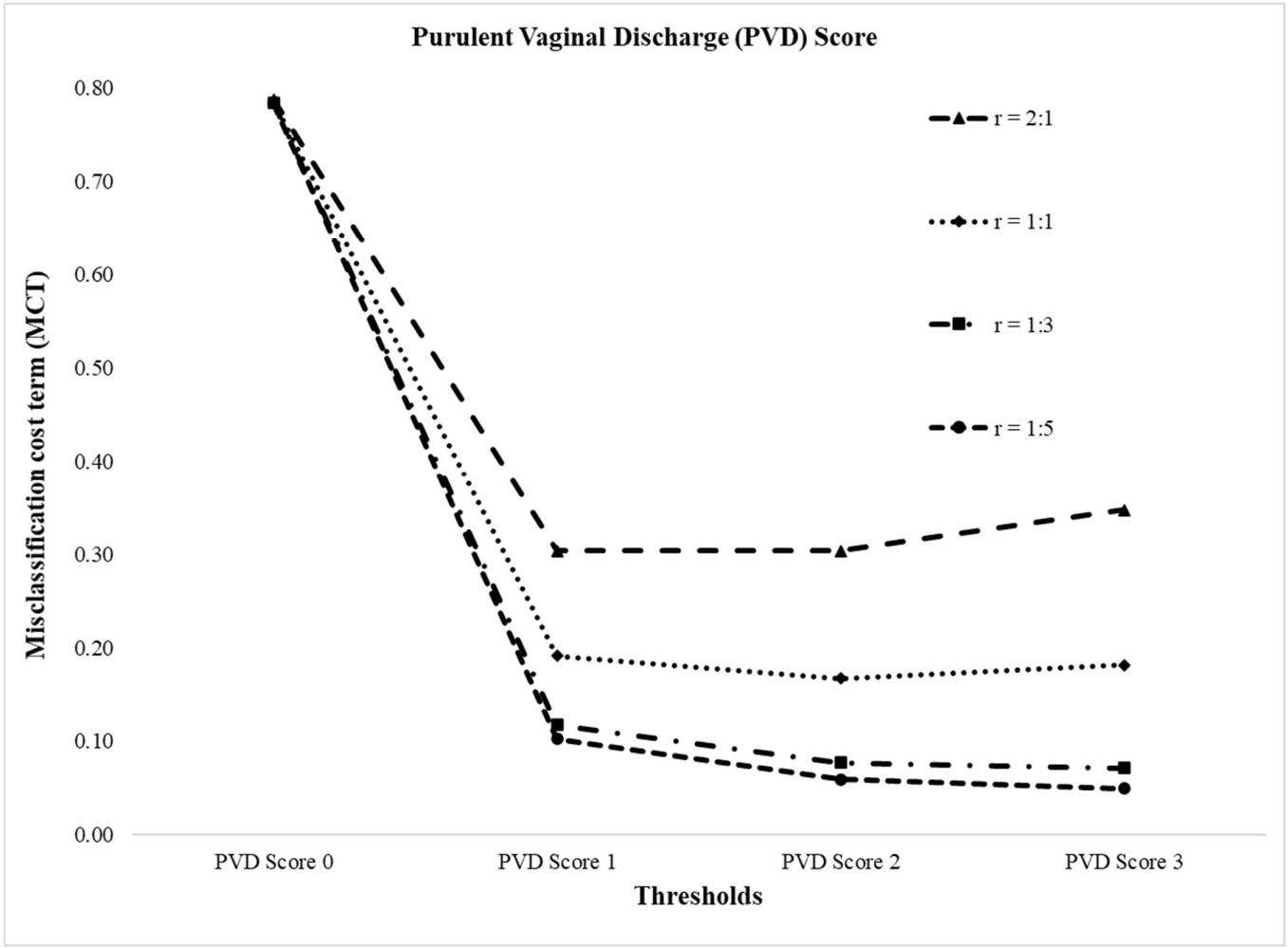

Figure 3.7 Misclassification cost term (MCT) at different thresholds and for different false negative to false positive costs ratio of purulent vaginal discharge (PVD) score. The MCT = (1-*p*) * (1-Sp) + (*r***p*) * (1-Se) is calculated on based median posterior inferences for each test Se and Sp (from Table 3.5), *r* is the of false negatives to false positives costs ratios (2:1, 1:1, 1:3 and 1:5) and *p* is a fixed prevalence of 20%. The median posterior inferences for each test were obtained using a Bayesian latent class analysis for the detection of reproductive tract disease at the pre-breeding examination in Irish dairy cows (n=2460) in 8 herds who were between 25-86 DIM. In this figure, PVD varying from scores 0 to 3 with UE fixed at ≥ score 1 (i.e., trace intraluminal fluid).

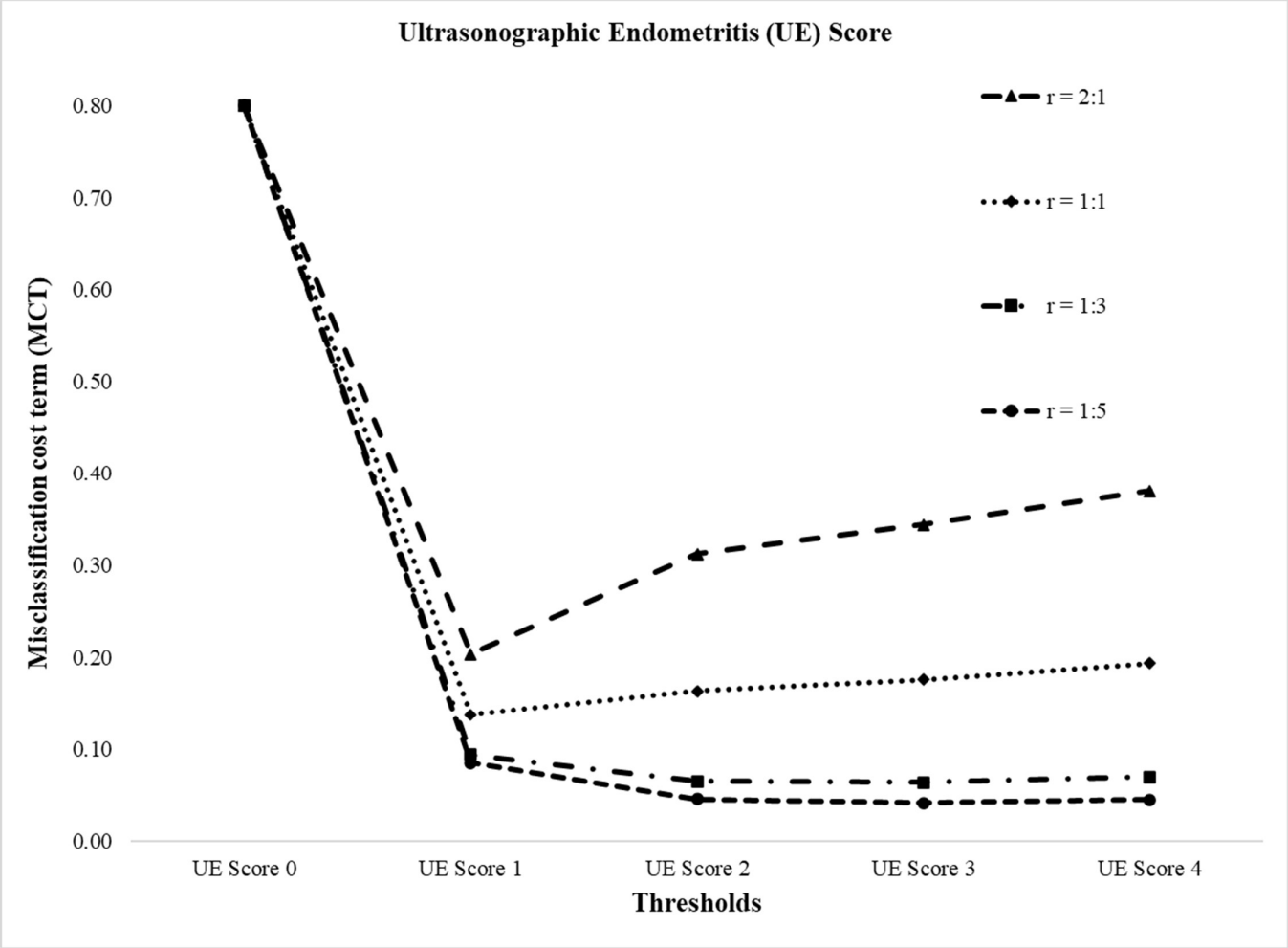

Figure 3.8 Misclassification cost term (MCT) at different thresholds and for different false negative to false positive costs ratio of ultrasonographic endometritis (UE) score. The MCT = (1-*p*) * (1-Sp) + (*r**p*) * (1-Se) is calculated on based median posterior inferences for each test Se and Sp (from Table 3.5), *r* is the of false negatives to false positives costs ratios (2:1, 1:1, 1:3 and 1:5) and *p* is a fixed prevalence of 20%. The median posterior inferences for each test were obtained using a Bayesian latent class analysis for the detection of reproductive tract disease at the pre-breeding examination in Irish dairy cows (n=2460) in 8 herds who were between 25-86 DIM. In this figure, UE varies from scores 0 to 4 while PVD is fixed at ≥ score 1 (i.e. flecks of pus).

Table 3.6. Final Bayesian latent class model for the detection of reproductive tract disease at the pre-breeding examination in Irish dairy cows (n=2460) between 25-86 days in milk in 8 herds. Purulent vaginal discharge (PVD) score is at a threshold of score ≥ 1 (i.e., flecks of pus) and ultrasonographic endometritis (UE) score is at a threshold of score ≥ 1 (i.e., trace intraluminal fluid, ≤2mm). Test median sensitivity, specificity, within herd prevalence, positive predictive (PPV) and negative predictive values (NPV) are illustrated alongside a 95% Bayesian probability interval (BPI).

Trait	Parameter	Median Estimate (95% BPI)	PVD PPV (95% BPI)	PVD NPV (95% BPI)	UE PPV (95% BPI)	UE NPV (95% BPI)
PVD score [a]	Sensitivity	0.44 (0.29-0.60)	-	-	-	-
	Specificity	0.90 (0.86-0.93)	-	-	-	-
UE score [b]	Sensitivity	0.67 (0.33-1.00)	-	-	-	-
	Specificity	0.91 (0.83-1.00)	-	-	-	-
Within herd prevalence [c]	Herd 1	0.14 (0.07-0.22)	0.41 (0.14-0.70)	0.91 (0.89-0.94)	0.56 (0.13-1.00)	0.94 (0.94-1.00)
	Herd 2	0.17 (0.08-0.26)	0.46 (0.16-0.75)	0.89 (0.87-0.93)	0.61 (0.15-1.00)	0.93 (0.93-1.00)
	Herd 3	0.21 (0.11-0.35)	0.53 (0.22-0.82)	0.86 (0.81-0.90)	0.67 (0.20-1.00)	0.91 (0.91-1.00)
	Herd 4	0.17 (0.09-0.27)	0.46 (0.18-0.76)	0.89 (0.86-0.92)	0.61 (0.17-1.00)	0.93 (0.92-1.00)
	Herd 5	0.27 (0.17-0.38)	0.61 (0.31-0.84)	0.81 (0.79-0.85)	0.74 (0.29-1.00)	0.88 (0.86-1.00)
	Herd 6	0.21 (0.11-0.31)	0.53 (0.21-0.79)	0.85 (0.84-0.91)	0.67 (0.20-1.00)	0.91 (0.91-1.00)
	Herd 7	0.24 (0.15-0.35)	0.58 (0.28-0.82)	0.83 (0.81-0.87)	0.71 (0.26-1.00)	0.89 (0.87-1.00)
	Herd 8	0.18 (0.09-0.26)	0.47 (0.17-0.75)	0.88 (0.87-0.92)	0.62 (0.16-1.00)	0.93 (0.93-1.00)

Table 3.6 Footnotes

[a] = Informative priors for PVD Se [beta (12.2, 7.0)] and Sp [beta (42.6, 5.6)].

[b] = Flat (i.e., non-informative) priors [beta (1,1)] for UE Se and Sp.

[c] = Informative priors for within herd prevalence [beta (5.2, 17.6)].

Table 3.7. Sensitivity analysis of the final Bayesian latent class model for the detection of reproductive tract disease at the pre-breeding examination in Irish dairy cows (n=2460) in 8 herds using purulent vaginal discharge (PVD) score ≥ 1 (i.e., flecks of pus) and ultrasonographic endometritis (UE) score ≥ 1 (i.e., trace intraluminal fluid, ≤2mm) as positive for target condition.

Dataset	n	DIM Range	Trait	Parameter	Median Estimate	95% BPI
Final [a]	2460	25-86	PVD	Sensitivity	0.44	0.29-0.60
				Specificity	0.90	0.86-0.93
			UE	Sensitivity	0.67	0.33-1.00
				Specificity	0.91	0.83-1.00
Flat priors [b]	2460	25-86	PVD	Sensitivity	0.24	0.02-0.45
				Specificity	0.85	0.79-0.91
			UE	Sensitivity	0.47	0.00-0.81
				Specificity	0.88	0.76-0.98
Final model subset by DIM at PBE [c]	961	25-45	PVD	Sensitivity	0.59	0.45.-0.74
				Specificity	0.87	0.82-0.91
			UE	Sensitivity	0.87	0.61-1.00
				Specificity	0.90	0.82-0.97
Final model subset by DIM at PBE [c]	1499	46-86	PVD	Sensitivity	0.44	0.29-0.61
				Specificity	0.94	0.91-0.97
			UE	Sensitivity	0.36	0.07-0.71
				Specificity	0.89	0.81-0.97

Table 3.7 Footnotes

[a] = Final Bayesian latent class model output for Se and Sp for PVD and UE using informative priors for PVD and within herd prevalence and non-informative priors for UE.

[b] = Final Bayesian latent class model output for Se and Sp for PVD and UE using flat (i.e., non-informative) priors for PVD and for UE

[c] = Subdivision of the final data set by DIM at PBE (25-45 DIM and 46-86 DIM) to examine if the DIM at examination influenced the posterior inferences in the final model.

3.5 Discussion

In this retrospective observational study, RTD status was determined by two practical cow side diagnostic tests, PVD scoring using a Metricheck device and UE scoring using transrectal ultrasonography of the reproductive tract. We examined the association of PVD or UE score at the PBE on the hazard of pregnancy within the subsequent breeding season in a select group of Irish seasonal calving pasture-based dairy cows. Additionally, we determined, using a Bayesian latent class model, which assumed conditional dependence between the tests, the test Se and Sp of PVD or UE scoring for the detection of RTD at the time of the PBE in the same population of cows. Both objectives were important to investigate as they have practical implications for decisions relating to cow-side diagnostic screening and potentially treatment for RTD in seasonally calving pasture-based dairy cows.

In this study, cows with higher PVD or UE scores were less likely to conceive than cows within the normal or referent categories (i.e., PVD or UE score of 0). This agrees with studies in seasonal systems that have examined the impact of RTD on reproductive outcome, which have examined either PVD (McDougall et al., 2007; Giuliodori et al., 2013; Giuliodori et al., 2017) or UE scoring (Mee et al., 2009; Šavc et al., 2016). Subsequent reproductive outcome is impaired in cows suffering with RTD as the infection and inflammation results in an impaired endometrium, slower growth of the dominant follicle, lower circulating estradiol concentrations and thus, a lower probability of ovulation (Sheldon et al., 2002b). These effects are mediated through the release of host cytokines in response to infections and bacterial lipopolysaccharide which directly damages the endometrium and indirectly disrupts ovarian steroidogenesis and follicular growth (Herath et al., 2007; Herath et al., 2009). In a seasonal spring-calving herd, the effects of these processes manifest as a reduction in the probability of submission for an AI, as estrus expression is either absent or reduced, and/or a reduction in the probability of conception due to impaired oocyte function or endometrial damage. In the present study, the initial descriptive statistics implied that the mean proportion of cows submitted for AI within 24 days after EESD as well as the mean first serve pregnancy per AI were both negatively impacted as a function of increasing score of each PBE test result (i.e., PVD or UE scores) and this is a consistent finding found in research conducted in other seasonal systems were it appears to impact both submission for AI and probability of conception to AI (McDougall et al., 2007; McDougall et al., 2020).

Although numerous studies have shown that RTD status impacts future reproductive performance, the threshold score when this performance is affected does differ between the present study and other research from seasonal systems (McDougall et al. 2007; Giuliodori et al. 2013; Giuliodori et al. 2017). In the latter studies, flecks of purulent material (PVD scores of ≥ 1) detected at the PBE have been shown to have a negative impact on subsequent reproductive performance. In the present study, the PVD scores of 2 or 3 (i.e., $\geq$ mucopurulent discharge) were associated with impaired reproductive performance, similar to early research in populations of dairy cows in year-round systems (LeBlanc et al. 2002; Dubuc et al., 2010). However, based some of the insight gained from the predicted probability

plots from the final PVD model, it is likely that the threshold over which there is impaired reproductive performance is influenced by when the disease is present relative to a cow's EESD (which in seasonal herds, is herd MSD for the majority of cows).The present study's predicted probability plots from the PVD model suggested that if flecks of purulent material are diagnosed at the PBE but the cow is greater than 3 weeks from breeding (i.e., her EESD) then the impact this score has on subsequent reproductive performance is negligible. However, if cows have flecks of purulent material or worse present closer to the time of breeding (i.e., <3 weeks to EESD) then it is implied that the subsequent reproductive performance may potentially be impaired. McDougall et al., (2020), recently reported that cows with flecks of purulent material present did not have poorer reproductive performance than those with clear mucous and this was based when only PBEs were performed on average 29.5 days before herd MSD. These results are similar with our work, if only the cohort of dairy cows examined greater than 3 weeks before EESD are considered. The reason for these finding may well be that cows with less severe signs further away from the breeding period have a greater time period for recovery and thus subsequent fertility may not be impacted yet, something which is not the case for those cows with signs of disease present close to breeding commencement. Although further validation studies are likely required for confirmation, based on the results of the present study, we would suggest a threshold of PVD score ≥2 (i.e. ≥ mucopurulent) may be appropriate at all times. However, if PBEs are taking place closer to the breeding (e.g. in < 3 weeks to breeding), then lowering the threshold to PVD score ≥1 (i.e. ≥ fleck of purulent material) may be an appropriate approach.

In the present study, UE scores of 1, 2, 3 or 4 were associated with impaired reproductive performance. Based on our predicted probability plots from the final UE model, it is clear that across each period a UE score of ≥1 had impaired reproductive performance when compared to UE score 0 across all the time period relative to EESD. However, like in the PVD model, the predicted impact of these scores on reproductive performance tended to be diminished in the lower scores that were further away from EESD. Although further validation studies are likely required for conformation, based on the results of the present study, we would suggest a threshold of UE score ≥1 would be appropriate for treatment if cows are being examined at all time points relative to breeding commencement but consideration must be made when lower scores are detected and the PBE is taking place further away from breeding as this impact on subsequent fertility may be marginal.

Additional factors that may diminish reproductive performance in dairy cows in seasonal systems were also highlighted in the present study. In the multivariable Cox proportional hazards model, other factors were associated with a decreased likelihood of pregnancy and included, increasing parity, poor BCS at the PBE, inferior genetic merit for fertility and lower DIM at commencement of breeding. This agrees with other research in seasonal systems that has noted poorer subsequent reproductive performance in cows experiencing BCS loss (Roche et al., 2007), cows of advanced parity, cows with inferior genetic merit and, later calving cows (Carty et al., 2020). In an era where antimicrobial stewardship is in much

sharper focus, it is likely that a more holistic approach whereby some or all these factors will be considered in conjunction with the time relative to breeding and the diagnostic score severity to assist veterinarians decision-making process when considering the value in pursuing antimicrobial therapy. This will likely be a fruitful avenue of further research.

A reasonable challenge facing farmers and veterinarians is whether to use a diagnostic test to screen a herd for a particular disease. Additionally, the choice of the type of test, the number of tests and the testing strategy are as an important consideration. In the case of RTD, to arrive at this conclusion, both the veterinarian and farmer must consider factors such as the economics of the condition, the cost and practicality of screening for disease and the cost benefit analysis of treatment. Herd disease prevalence and diagnostic test parameters such as, Se, Sp, positive and negative predictive values are thus important to estimate to enhance decisions relating to whether screening is warranted and how it should be performed. The present study examined both diagnostic tests at a variety of thresholds using a Bayesian latent class model to determine these test parameters at the PBE and attempted to determine an optimal threshold for detection of RTD at that time point.

Based on our Bayesian latent class model, it is apparent that the optimal threshold for PVD and UE scoring was ≥ score 1. This choice was based on the tests Se, Sp and convergence of the MCT values attained from different simulations of cost-benefit with a similar approach as to what has been described by others (Arango-Sabogal et al., 2019). This was somewhat contradictory to what was found based on some of our multivariable Cox proportional hazards models especially in relation to PVD, where a PVD score ≥ 2 was considered to impair subsequent reproductive performance. However, this contrast is likely since the Cox proportional hazards model is based on an outcome that is in the future and is dependent on multiple other variables, including considerations such as time of the diagnosis relative to the EESD which has been discussed above as a potential reason for variation in thresholds selection. By comparison to Cox proportional hazards model, the Bayesian latent class model approach is based on disease status at the moment of testing. This so-called 'paradox' in thresholds has been previously noted by McDougall et al. (2020) and is important to remember when interpreting the results of our study.

Similar to other research on the test parameters for cow-side diagnosis of RTD (Arango-Sabogal et al. 2020; McDougall et al., 2020), we found that for both tests that Sp was relatively high (>85%), while Se was found to be generally poor (<50%) to moderate (50-70%). This low Se means that many cow-sides tests will fail to detect many cases of RTD which can be frustrating for farmers and veterinarian alike. At our selected optimal thresholds and based on the Bayesian latent class model, median PVD test Se and Sp were 44% (95% BPI: 29-60%) and 90% (95% BPI: 86-93%), respectively while median UE test Se and Sp were 67% (95% BPI: 33-100%) and 91% (95% BPI: 83-100%). Recent research using similar methodology has estimated PVD scoring at the same threshold (i.e., ≥ flecks of pus) as having a median Se and Sp of 70.7% (95% BPI: 46.8–93.4%) and 88.1% (95% BPI: 81.3–95.4%) (McDougall et al., 2020) or 64.2% (95% BPI: 59.1–70.3) and 96.6% (95% BPI: 91.3–99.8%) (Arango-Sabogal et

al., 2019). The present study's PVD Sp estimate is broadly comparable, yet our Se estimate is below previously reported work. Reasons to explain the difference might include variations in the study populations, the modelling approach, the variation in DIM when the PBE was performed across studies and/or the prior assumptions included in our Bayesian latent class model. To examine the effect of some of these factors, we did conduct a sensitivity analysis. As part of the sensitivity analysis, we subdivided the dataset based on DIM at PBE (Table 3.7). In terms of PVD Se estimate, when the dataset examined only PBEs based on a DIM range of 25-45 DIM, it was comparable to other research [28-42 DIM; Arango-Sabogal et al. (2020)] as there was a convergence of our median Se estimate toward that of Arango-Sabogal et al. (2020); 59% (95% BPI; 45-74%) versus 64% (95%; BPI; 59.1–70.3%) in the former study. Some of these differences in the present study may at least in part explain some of the variation in PVD score Se estimates when comparing between studies. It also highlights the important point that test Se is likely dependent on DIM at the PBE.

Previous work on estimating Se and Sp for UE scoring has been conducted by a handful of authors with variable results. Barlund et al. (2008) estimated UE score with a threshold of >1 mm intraluminal fluid present as having a Se and Sp of 38.5% (95% CI: 19.8-57.2%) and 78% (95% CI: 72.1-83.8%), respectively while Meira et al. (2012) estimated Se and Sp for the presence of any intraluminal fluid as 88% (95% CI: 62-99%) and 62% (95% CI: 48-74%), respectively. Our present study's median Se estimate is somewhat in between these studies at 67%, but our Sp estimate is higher than both of those studies at 91%. However, it is worth noting some differences between these studies and the present work. In the present study, Se and Sp were estimated using a Bayesian latent class model which assumed that neither test was perfect nor adjusted the estimates of Se and Sp accordingly (Pouillot et al., 2002). In the case of both Barlund et al. (2008) and Meira et al. (2012), both used the reference method of endometrial cytology to determine test Se and Sp. Additionally, the threshold for positivity on the endometrial cytology (which was the standard reference test) differed between these researchers (>8% polymorphonuclear cells at 28-41 d postpartum in Barlund et al., 2008; >18% polymorphonuclear cells at 21-33 d postpartum or >10% polymorphonuclear cells at 34-47 days in Meira et al., 2012) as did their Se and Sp estimates (Se = 38.5% vs 88%; Sp = 78% vs 62%). Latent class models are useful in any disease where no standard diagnostic test exists (van Smeden et al., 2014) and an application of a Bayesian approach is applicable as parameters are estimated by the observed data while also being informed by prior research estimates (Kostoulas et al., 2017). In the case of UE scoring, we deemed this prior research on Se and Sp (Barlund et al., 2008; Meira et al. 2012) may not have been useful for our model based on the reference method used. Therefore our prior assumptions were kept as flat throughout the modelling.

As previously noted, the sensitivity analysis in the present study seemed to indicate that the DIM at PBE influenced test Se estimates for PVD scoring but it had an even more profound influence on UE scoring. When the dataset examined only PBEs based on a DIM range of 25-45 DIM, UE scoring had a median

(95% BPI) Se estimate of 87% (95% BPI: 61-100%) while when only PBEs based on a DIM range of 46-86 DIM were examined the median Se estimate decreased to 36% (95% BPI: 7-71%). This may not be as stark a finding as it first seems as it is well documented that as DIM increases, the probability of detection of RTD decreases (Gautam et al., 2010; Giuliodori et al., 2017; Kelly et al., 2020). This decrease in detection is largely assumed to be due to apparent self-cure (Dubuc et al., 2010; Giuliodori et al., 2017) or a shift from the clinical state to a subclinical state (Kelly et al., 2020) which could be more difficult to detect with the cow-side diagnostic tests examined in the present study. In year round calving dairy herds, PBEs typically happen prior to the end of the voluntary waiting period on a tight range of DIM based on the individual cow. However, in seasonal calving herds, PBEs take place on a set date or dates before the MSD which is typically sometime in the two months preceding breeding, i.e., either March, April or May in the Northern Hemisphere (Mee et al., 2009). It is for this reason that there is a wide variation in DIM at PBE noted in this study, but it is also why modelling the Se and Sp estimate was conducted based on the complete range of DIM at PBE as it is a more representative of what would be encountered by veterinarians working with seasonal calving systems. However, it is useful for the veterinarian to understand, that based on the present study, visiting the farm closer to the MSD is likely to decrease the Se of both diagnostic tests, assuming there is not a poorly distributed calving pattern on farm.

Some of the limitations of our retrospective observational study on RTD have already been addressed and methods to mitigate these issues discussed. Firstly, the convenience sampling used and the number of used herds in the present study, might limit the inferences that can be made around reproductive outcome or test parameters conclusions. Additionally, in any retrospective study, no adjustments to the study population or design can be made. In the case of the present study, treatment of cows with PVD or UE scores of ≥ 2 was part of farm management decision and thus may have influenced reproductive outcome results, although despite this, reproductive outcome was still not improved to the level of scores 0 or 1 in both cases and these cows did not receive treatment. This treatment would also have had no effect on the Bayesian latent class model part of the research as test parameter estimates were based on a latent class of RTD at the time of the PBE and not on the reproductive outcome.

That said, one of the assumptions of Bayesian models is that there is constant accuracy of diagnostic tests over time and operators but in the present study, it was not possible to validate the latter assumption. As previously mentioned, the alterations in test Se and Sp estimates noted by separately analyzing the two categories of DIM at PBE might indicate that the constant accuracy of diagnostic tests over time was not a valid assumption and that use of this methodological approach may warrant separate analysis based on a tighter range of DIM at PBE.

3.6 Conclusions

Reproductive tract disease in early lactation has a significant impact on subsequent reproductive performance in seasonal calving pasture-based dairy cows. It can be diagnosed by multiple methods, two of these methods are relatively cheap and rapid in their diagnosis and include assessing vaginal discharge for the presence of purulent material or transrectal ultrasonography of the reproductive tract. Scoring systems which classify cows at varying degrees of severity of disease are useful in predicting subsequent reproductive performance and may play a role in planning therapeutic decisions in cows. Dairy cows that have either ≥ mucopurulent discharge (PVD score of ≥ 2) or ≥ trace intraluminal fluid (UE score of ≥ 1) were associated with impaired subsequent reproductive performance but consideration of the time of diagnosis relative to the commencement of breeding should also be considered when determining a threshold to treat cows. Determining important diagnostic test parameters, such as, sensitivity and specificity, in conjunction with the estimated within herd prevalence is important to inform decisions related to disease screening and testing strategies on farms. Based on our Bayesian latent class model, the optimal threshold to detect reproductive tract disease at the pre-breeding examination was either ≥ flecks of purulent material in discharge (PVD score of ≥ 1) or ≥ trace intraluminal fluid (UE score of ≥ 1). At these thresholds, transrectal ultrasonography appeared to have better test sensitivity and specificity, although both tests are likely affected by days in milk at pre-breeding examination with greater test sensitivity achieved when examination takes place at earlier days in milk.

3.7 Supplementary Materials

Table 3.8. Univariable Cox proportional hazards model assessing the associations between each available explanatory variable on the hazard of pregnancy within a breeding season. 4756 PBEs were analyzed on 2338 cows in 8 Irish seasonally calving pasture-based dairy herds over the 5-year period.

Categorical Variables	Class	n	Hazard Ratio	95% CI	P-value
Farm	1	930	Referent		
	2	606	0.77	0.69-0.86	<0.01
	3	553	0.80	0.71-0.89	<0.01
	4	511	0.60	0.54-0.68	<0.01
	5	721	0.86	0.78-0.95	<0.01
	6	515	0.86	0.77-0.97	<0.01
	7	542	0.72	0.65-0.81	<0.01
	8	378	0.67	0.59-0.76	<0.01
Year	2014	510	Referent		
	2015	777	1.01	0.90-1.14	0.85
	2016	977	0.99	0.881.11	0.89
	2017	1161	1.06	0.95-1.19	0.31
	2018	1331	1.05	0.94-1.18	0.35
Parity	1	1476	Referent		
	2	1151	0.94	0.86-1.02	0.15
	3	823	0.82	0.75-0.91	<0.01
	4	569	0.77	0.69-0.86	<0.01
	5+	737	0.61	0.55-0.68	<0.01
Breed	Holstein Friesian	3659	Referent		
	Jersey	608	0.97	0.89-1.07	0.58
	Crossbred	189	1.24	1.06-1.44	<0.05
Calving Difficulty Score	1 = No assistance	4247	Referent		
	2 = Some assistance	364	0.95	0.84-1.06	0.34
	3 or 4 = Considerable or veterinary assistance	145	0.87	0.72-1.04	0.13
Twins	No	4662	Referent		
	Yes	94	0.75	0.59-0.94	<0.05
RFM	No	4677	Referent		
	Yes	79	0.78	0.61-1.00	0.05
Stillbirth	No	4570	Referent		
	Yes	186	0.89	0.76-1.04	0.14
Single Calf Sex	Female	2381	Referent		
	Male	2281	1.03	0.97-1.10	0.33
Sire Type	Dairy	4105	Referent		
	Beef	651	1.06	0.95-1.18	0.29
Calving BCS	Ideal (3.0-3.25)	3992	Referent		
	Fat (≥3.5)	539	1.06	0.97-1.17	0.20
	Thin (<3)	225	0.81	0.70-0.94	<0.01
BCS at PBE	Ideal (2.75-3.25)	4363	Referent		
	Fat (≥)	66	1.14	0.88-1.47	0.31
	Thin (<2.75)	327	0.68	0.59-0.77	<0.01
Corpus Luteum at PBE	Yes	3491	Referent		
	No	1265	0.92	0.86-0.99	<0.05

Table 3.8. Continued

Categorical Variables	Class	n	Hazard Ratio	95% CI	P-value
PVD Score	0	3959	Referent		
	1	448	0.93	0.84-1.03	0.18
	2	184	0.80	0.68-0.95	<0.01
	3	165	0.69	0.58-0.82	<0.01
UE Score	0	3753	Referent		
	1	685	0.88	0.81-0.97	<0.01
	2	158	0.69	0.58-0.83	<0.01
	3	89	0.76	0.60-0.97	<0.01
	4	71	0.37	0.28-0.51	<0.01
Time of PBE relative to EESD	≤3 weeks to EESD	2430	Referent		
	>3 weeks to EESD	1788	1.05	0.98-1.13	0.14
	After EESD	538	0.63	0.55-0.72	<0.01
305-Day Predicted Yield	1st Quartile	1189	Referent		
	2nd Quartile	1189	0.93	0.85-1.03	0.16
	3rd Quartile	1189	0.80	0.73-0.89	<0.01
	4th Quartile	1189	0.76	0.69-0.84	<0.01
PTA milk yield	1st Quartile	1189	Referent		
	2nd Quartile	1189	0.87	0.80-0.95	<0.01
	3rd Quartile	1189	0.88	0.81-0.96	<0.01
	4th Quartile	1189	0.92	0.84-1.00	0.05
PTA milk fat yield	1st Quartile	1189	Referent		
	2nd Quartile	1189	0.89	0.82-0.97	0.01
	3rd Quartile	1189	0.90	0.83-0.98	0.02
	4th Quartile	1189	0.91	0.83-0.99	0.03
PTA Calving Interval	1st Quartile	1189	Referent		
	2nd Quartile	1189	0.86	0.79-0.94	<0.01
	3rd Quartile	1189	0.82	0.75-0.89	<0.01
	4th Quartile	1189	0.63	0.58-0.69	<0.01
Continuous Variables	**Class**	**n**	**Hazard Ratio**	**95% CI**	**P-value**
DIM at EESD	-	4756	1.02	1.01-1.02	<0.01
305-Day Predicted Yield	-	4756	1.00	1.00-1.00	<0.01
PTA Milk Yield KGS	-	4756	1.00	1.00-1.00	0.05
PTA Milk Fat KGS	-	4756	0.99	0.99-1.00	0.05
PTA Calving Interval	-	4756	0.91	0.89-0.92	<0.01

Figure 3.9. Graphical examples the ultrasonographic classification system for ultrasonographic endometritis (UE) used in the present study. It was modified from Mee at al. (2009). UE score 0 = No intraluminal fluid or trace volume of anechoic (black) and a spoke-wheel-shaped lumen; UE score 1 = Trace volume (≤2 mm) of hyperechoic (white) intraluminal fluid in a spoke-wheel-shaped lumen with infolding of the endometrium; UE score 2 = Small volume (>2 to <5 mm) of intraluminal fluid of mixed echogenicity (grey or white); UE score 3 = Moderate volume (≥5 to <10 mm) of intraluminal fluid of mixed echogenicity (grey or white); UE score 4 = Large volume (≥10 mm) of intraluminal fluid of mixed echogenicity (grey or white). White arrows indicate intraluminal fluid.

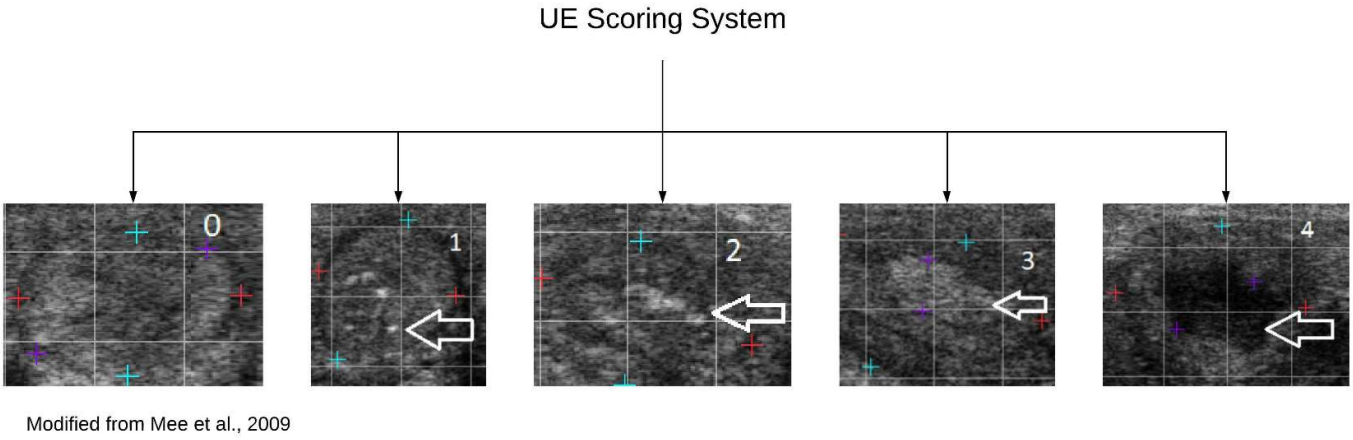

Table 3.9. Cross-tabulated results for the final Bayesian model for purulent vaginal discharge (PVD) score (Threshold = ≥ 1; flecks of pus) and ultrasonographic endometritis (UE) score (Threshold = ≥ 1; trace (≤ 2 mm) intraluminal fluid) used in the final model to estimate diagnostic test sensitivity and specificity.

Herd	PVD + (score ≥1) UE + (score ≥1)	PVD + (score ≥1) UE - (score 0)	PVD – (score 0) UE + (score ≥1)	PVD – (score 0) UE + (score 0)	Total
1	23	38	41	313	415
2	17	36	43	226	322
3	37	22	21	230	310
4	23	31	25	218	297
5	40	21	51	233	345
6	17	18	37	162	234
7	28	23	37	183	271
8	17	22	33	194	266
Total	202	211	288	1759	2460

Table 3.10 Checklist of items for the Standards for the Reporting of Diagnostic accuracy studies by the use of Bayesian Latent Class Models (STARD-BLCM) provided in Kostoulas et al. (2017).

Section	Item n#	STARD-BLCM
Title/Abstract/Keywords	1	Identification as a study of diagnostic accuracy, using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC) and Bayesian latent class models
Abstract	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)
Introduction	3	Scientific and clinical background, including the intended use and clinical role of the tests under evaluation
	4	Study objectives and hypotheses, such as estimation of diagnostic accuracy of the tests for a defined purpose through BLCM
Study Design	5	Whether data collection was planned before the tests were performed (prospective study) or after (retrospective study)
Participants	6	Eligibility criteria and description of the source population
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)
	8	Where and when potentially eligible participants were identified (setting, location, and dates)
	9	Whether participants formed a consecutive, random or convenience series
Test methods	10a	10.Description of the tests under evaluation, in sufficient detail to allow replication, and/or cite references
	10b	Reference standard, in sufficient detail to allow replication
	11	Rationale for choosing the tests under evaluation in relation to their purpose
	12a	12. rationale for test positivity cut-offs or result categories of the tests under evaluation, distinguishing pre-specified from exploratory
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory

Section	Item n#	STARD-BLCM
	13a	13. Whether clinical information was available to the performers or readers of the tests under evaluation
	13b	Whether clinical information and index test results were available to the assessors of the reference standard
Analysis	14	14a. BLCM model for estimating measures of diagnostic accuracy
		14b Definition and rationale of prior information and sensitivity analysis
	15	How indeterminate results of the tests under evaluation were handled
	16	How missing data of the tests under evaluation were handled
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory
	18	Intended sample size and how it was determined
Participants	19	Flow of participants, using a diagram
	20	Baseline demographic and clinical characteristics of participants
	21a	21. Not applicable: the distribution of the targeted conditions is unknown, hence the use of BLCM
	21b	Distribution of alternative diagnoses in those without the target condition
	22	Time interval and any clinical interventions between the tests under evaluation
Test results	23	Cross tabulation of the tests' results (or for continuous tests results their distribution by infection stage)
	24	Estimates of diagnostic accuracy under alternative prior specification and their precision (such as 95% credible/probability intervals)
	25	Report any adverse events from performing the of the tests under evaluation
Discussion	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability

Section	Item n#	STARD-BLCM
	27	Implications for practice, including the intended use and clinical role of the tests under evaluation in relevant settings (clinical, research, surveillance etc.)
	28	Registration number and name of registry
	29	Where the full study protocol can be accessed
	30	Sources of funding and other support; role of funders

Chapter 4

Estimation of the true prevalence of inaccurate artificial inseminations in Irish milk recording dairy cows using a Bayesian latent class analysis.

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4.0. Abbreviations used in this chapter

AC: Apparent conception

AI: artificial insemination

BLCM: Bayesian latent class models

BPI: Bayesian probability interval

ELISA: Enzyme-linked immunosorbent assay

IAI: Inaccurate artificial insemination (Please note for the purposes of this thesis this is synonymous with **EDI**: Estrus detection inaccuracy described in chapter 5)

LA: Latex agglutination

LCM: Latent class model

LFT: Lateral flow enzyme immunoassays

MP4: Milk progesterone

P4: Progesterone

PA: Apparent prevalence

PT: True prevalence

RIA: Radioimmunoassays

SCC: Somatic cell count

Se: Test sensitivity

Sp: Test specificity

4.1 Abstract

Inaccurate artificial insemination (**IAI**) refers to an artificial insemination (**AI**) that is performed when a cow is not in estrus. IAIs have economic impacts on the dairy industry through semen wastage or iatrogenic pregnancy loss. However, few studies have quantified the prevalence of IAIs in a population. The primary objective of this prospective study was to estimate the cow-level true prevalence of IAIs in Irish milk recording dairy herds using a latent class model with a Bayesian framework. Milk samples were collected at a milk recording laboratory from 576 dairy cows in 125 herds who had received an AI on the same day they were sampled for routine milk constituent analysis. Milk progesterone (**MP4**) analysis was conducted on these samples using radioimmunoassay to determine the progesterone concentration. Fertility data (i.e., subsequent calving date) was retrospectively obtained from the Irish National Cattle Breeding Federation for milk sampled cows and an apparent conception (**AC**) to the sample AI was determined based on an estimated gestational range of 270-290 days. Both tests (MP4 and AC) were used in a latent class model to estimate the true prevalence of IAI. For the MP4 test, a concentration of ≥ 5ng/ml in whole milk was deemed to be test positive while for the AC test, a cow that did not conceive to the sampled AI was deemed test positive. Prior information for prevalence of IAI was obtained from a literature review while MP4 sensitivity (**Se**) and specificity (**Sp**) were obtained from expert opinion. Non-informative priors were used for the Se and Sp of the AC test. Posterior inferences (median and 95% Bayesian probability intervals; **BPI**) were obtained using the 'rjags' package in the R statistical software. In the final model, median cow-level true prevalence of IAI was 4.4% (BPI; 1.7–9.0%). Median Se and Sp estimates for MP4, were 83.0% (BPI; 65.0–96.2%) and were 97.4% (BPI; 94.6–99.6%), respectively. Median Se and Sp estimates for AC, were 64.8% (BPI; 44.5–88.6%) and 49.8% (BPI; 45.3–54.1%), respectively. The present study estimates that the overall cow-level true prevalence of IAI in Irish dairy cows is relatively low. This is the first study to report the cow-level true prevalence of IAI using a Bayesian latent class model.

4.2. Introduction

Inaccurate artificial insemination (**IAI**) (also termed 'false positive insemination') refers to an artificial insemination (**AI**) that is performed when a cow is not in estrus (Morton, 2004; Ingenhoff et al., 2016). These IAIs are costly for farmers and breeding companies as they are wasteful of technician time, semen and indeed, they have the potential to decrease fertility owing to the potential to disrupt an existing pregnancy (Appleyard and Cook, 1976; Sturman et al., 2000). Interference of existing pregnancies is particularly relevant in seasonally calving dairy systems, where conceiving early in the breeding season is paramount and additional opportunities to conceive are limited by the compact breeding period (McDougall, 2006).

Estimates of the prevalence of inaccurate estrus detection and subsequent IAIs from the literature show considerable variation, ranging from 3-4% (Rajamahendran et al., 1990; Ingenhoff et al., 2016) to almost ten times that amount in particular study cohorts (Oltner and Edqvist, 1981; Nebel et al. 1987). As there is no single gold standard test to determine an IAI, most studies have estimated the prevalence of IAIs by measuring progesterone concentrations, in either blood or milk (Dobson et al, 1975), in a single sample taken at the time of AI. This relies on the fact that around estrus, cows have a low circulating progesterone, while during the luteal phase of their cycle, they have high circulating progesterone (Ingenhoff et al., 2016). However, the use of P4 to determine whether the cow is truly in estrus has limitations as not all cows with low circulating progesterone (i.e., those in anestrus/periestrus) will have ovulation pending.

Many types of tests have been performed to determine the progesterone concentration in blood or milk and include; radioimmunoassays (**RIA**) (Heap et al., 1976), enzyme-linked immunosorbent assay (**ELISA**) (Arnstadt and Cleere, 1981), latex agglutination (**LA**) (Romagnolo and Nebel, 1993) and lateral flow enzyme immunoassays (**LFT**) (Waldmann and Raud, 2016).The differences in the various study populations, the biological samples tested (i.e., blood/whole milk/milk fat), and the laboratory methods of progesterone determination explain much of the variation noted. However, the threshold concentrations of progesterone that determines a sample as inaccurate and discrepancies in test sensitivity (**Se**) and specificity (**Sp**) are also likely to play an important role.

When any test is used to estimate true prevalence (**PT**), the apparent prevalence (**PA**) must be adjusted for the test Se and Sp (Messam et al., 2008). Failure to do so can result in a marked variation in PT estimation, as it common for test Se and Sp estimates to differ among study populations (Greiner and Gardner, 2000). Both frequentist and Bayesian methods have been described to estimate PT from PA (Messam et al., 2008). Frequentist approaches are straightforward to apply, assume a fixed proportion in a population and have been the more traditional way of estimating PT after adjusting for test Se and Sp (Messam et al., 2008). However, use of the Bayesian approach for the estimation of herd and animal-level PT has gained popularity in the last 20 years (Branscum et al., 2004). When this approach is used,

parameters are considered as random variables that can be modelled using probability distributions (McAloon et al., 2016). Additionally, Bayesian methods are useful in instances when sample sizes are small, prevalence is low and when unpredictable test Se and Sp must be included in the calculations of PT (Messam et al., 2008).

Latent class models (**LCM**) can be used in situations where no gold standard reference test exits (van Smeden et al., 2014). Applying a Bayesian approach to LCMs (**BLCM**) has been implemented across a number of areas of veterinary research (Branscum et al., 2004; Branscum et al., 2005) but is particularly useful when parameters with the LCM cannot be estimated by the observed data alone (Kostoulas et al., 2017).

The main objective of this present study was to use two classes of the observed data: 1.) milk progesterone (**MP4**) concentration on the day of AI and 2.) apparent conception (**AC**) to the sampled AI, to estimate the cow-level PT of IAIs in Irish milk recording dairy herds using a latent class model with a Bayesian analytical approach.

4.3. Materials and Methods

4.3.1. Study design

The present study was a prospective study which was based on a dataset of milk samples that were collected from cows who were milk recorded for milk constituent analysis (as part of routine management) on the same day they received an AI from a technician. Sample size was calculated prior to sampling and was based on expected apparent prevalence of 20% with a precision of 0.025 and 95% confidence interval (n=984) (Epitools; © Ausvet 2021; https://epitools.ausvet.com.au/). The apparent prevalence of 20% was based on the previous Irish work by White and Sheldon (2001) which milk sampled cows inseminated by technicians at the time of AI (Table 4.1). After data collection, as our sample size was lower than expected, and the accuracy of our MP4 test was unknown, a Bayesian latent class analysis (Messam et al., 2008) was deemed a useful alternative to estimate the cow-level PT of IAI. Thus the manuscript was written along the guidelines standards for the reporting of diagnostic accuracy studies that use BLCMs (Kostoulas et al., 2017; see Chapter 3, Section 3.7, Table 3.9).

4.3.2. Participants: Eligibility criteria and description of the source population

The study population were cows that were milk recorded on the day of AI and sampled for progesterone analysis as outlined below. Thus, the source population sampled from were cows in herds that routinely milk record and use an AI technician. As the milk sampling was conducted at a single milk recording laboratory (Irish Milk Laboratories Ltd.; www.imlabs.ie) in Bailieborough, (County Cavan, Ireland; 53°55′N, 6°58′W) and as this laboratory receives milk samples from dairy herds based in the western, northern and eastern part of the Republic of Ireland, only cows in this geographical region were sampled. Approximately, 94% (542/576) of the cows sampled had calved in the spring (January to May 2018) prior to sampling and thus most cows were likely part of seasonally calving dairy herds that predominate in Ireland (Veerkamp et al., 2002), although it is possible some cows were from herds with spilt-calving or year-round calving as these herds details were not available to the authors. All sampled cows were eligible for inclusion in the study.

4.3.3. Test Methods

4.3.3.1. Milk samples collection and analysis for progesterone concentration (Test 1)

Between 23rd of April to 23rd of June 2018, online access to AI technician data was granted by Progressive Genetics (www.progressivegenetics.ie). This period of the year is the typical artificial breeding period for majority seasonal calving pasture-based dairy herds in Ireland (Veerkamp et al., 2002) so was targeted for sampling. During this period, the milk recording laboratory was visited three days a week. All milk samples delivered to this laboratory had been taken by trained milk recording technicians and were a composite sample of whole milk and were being routinely tested for milk constituents (i.e., milk fat, protein, lactose, and **SCC**) using mid-infrared spectrometry. At this

laboratory, once a herd was identified as using an AI technician from the company, all cows that had received an AI on the same day as their routine milk recording were selected for milk sampling. Once a cow was selected for sampling for the present study, their individual milk sample, that was submitted to the laboratory for routine constituent analysis was identified from all the herd milk samples. The sample was removed, warmed in a water bath at 37^{O}C for 3 minutes, agitated and a 2ml aliquot was removed and placed in 5 ml labelled universal containers. Samples were then frozen at −20°C until subsequently analysed. All collected samples were analysed for milk progesterone (**MP4**) concentrations using a validated solid-phase RIA (DIAsource PROG-RIA_CT Kit; DIAsource ImmunoAssays S.A., Rue du Bosquet,2, B-1348 Louvain-la-Neuve, Belgium) as previously described by Kelly et al. (2021) (see Chapter 5). The results were quantitative and expressed as progesterone in nanograms per millilitre (ng/mL) of milk. Values over a threshold of ≥ 5ng/ml were considered as positive test results (i.e., an AI was deemed inaccurate) and used in the initial BLCM (Figure 4.1).

4.3.3.2. Fertility data collection and analysis for apparent conception to sampled AI (Test 2).

Data obtained from the genetics company at the time of milk sample collection included, cow and herd identification number, date of AI, AI technician identification number, sire code and whether the AI was a first or a repeat insemination conducted by the technician. Additionally, the Irish Cattle Breeding Federation (ICBF; www.icbf.ie) provided cattle breeding information on cows sampled as part of this research. This anonymised animal data was provided at a later point (November 2019) relative to the milk sample collection. The data included previous (2017 or 2018) and subsequent calving dates (2018-2019), parity, breed, departure, or deceased date if applicable, 305 day predicted milk yield, test day milk, fat, protein and lactose yield and somatic cell count (SCC) as well as genetic information such as the individual economic breeding index value and their various sub-indices values. Once all milk samples were assayed for progesterone the above data was then collated for descriptive statistics and for the BLCM. As no pregnancy data following AI was available for the cows but subsequent calving was available, an apparent conception (AC) was calculated for each cow based on whether calving occurred within 270-290 days from the sampled AI. For those cows that subsequently calved with a gestation range of 270-290 days from the sampled AI, the cow was deemed to be in estrus at insemination (i.e., 'negative' test result). Those that did not calve within the gestation range 270-290 were considered as a 'positive' test result (i.e., an AI was deemed inaccurate).

4.3.4. Target condition and Se and Sp definitions

The target condition for the present study were cows that were not in estrus at AI. This condition includes cows that are in the luteal phase of their estrous cycle, pregnant, in anestrus or in periestrus. In terms of the test methods used, the test Se referred to cows not in estrus and identified as such by a test while test Sp referred to cows that were in estrus and identified as such by a test. In the case of the MP4 test, test

Se was defined as the proportion of cows that were not in estrus at AI and were detected with elevated progesterone while test Sp was defined as the proportion of cows that were in estrus at AI and were detected with low progesterone. In the AC test, test Se was defined as the proportion of cows that were not in estrus at AI and did not conceive, while test Sp was defined as the proportion of cows that were in estrus at AI and did conceive. Figure 4.1 illustrates how the tests under evaluation relate to the target condition.

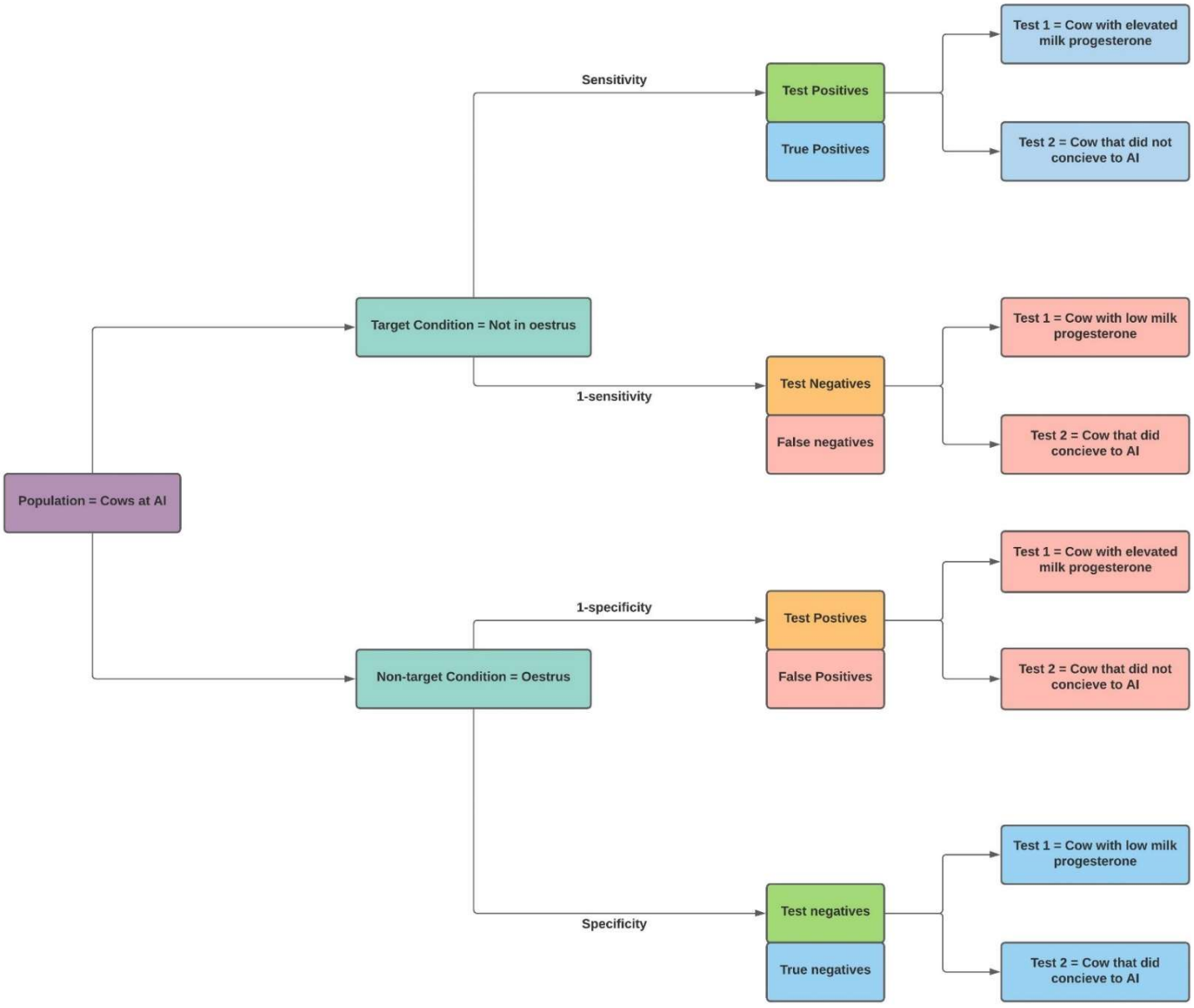


Figure 4.1 A flow diagram to illustrate how the tests under evaluation relate to the target condition.

4.3.5. Rationale for choosing the tests under evaluation in relation to their purpose.

Both tests (MP4 and AC) under evaluation were used to determine whether an AI was performed incorrectly or not. Elevation of MP4 is associated with the presence of a corpus luteum of the luteal phase of the estrous cycle or pregnancy and is what has been used in many studies to estimate the prevalence of IAIs (Nebel et al., 1987; Sturman et al., 2000). Although there are a multitude of factors that can influence whether a cow conceives and subsequently calves to a particular AI, those cows that are inseminated when not in estrus should not conceive and thus are less likely to calve within a normal gestation period following an incorrect AI. Cows that are not in estrus will be in the luteal phase of their estrous cycle, pregnant, in anoestrous or in periestrus. As a result, if a cow is inseminated when not in estrus, they may have conceived earlier (i.e., if already pregnant) or later (i.e., if in the luteal phase or anestrus) and thus should have a greater tendency to have a gestational range that is outside of the normal range by comparison to a cow inseminated when in estrus. Although, AC is not strictly a diagnostic test and is retrospective, the purpose for using it in this present study was to assist in the estimation of the cow-level PT of IAI and estimation of Se and Sp was deemed less important.

4.3.6. Definition of and rationale for tests positivity cut-offs

For the BLCM thresholds of ≥ 5ng/ml and calving within 270-290 days from sampled AI were used for MP4 and AC, respectively. Thresholds of 5 ng/ml in milk have been used as a threshold for the presence of luteal tissue by other researchers using whole milk (Nebel et al., 1987; White and Sheldon, 2001; Morton and Wynn, 2010) and cows are unlikely to be in estrus and conceive at this concentration. Cows that are inseminated when not in estrus should not conceive and thus tend to calve outside a normal gestation period following the sampled AI. Mean gestation length in Irish Holstein Friesians (i.e., the predominant dairy breed) is 279 days and ranges from 281-283 days for the next most popular breeds in Ireland (i.e., Jersey and Norwegian Red) (ICBF, 2020a). These mean gestation lengths are similar across the globe (Norman et al., 2009; Haile-Mariam and Pryce, 2019) with a range of 270-290 days considered normal (Foote, 1981). Hence, this gestation range was deemed most appropriate to calculate an AC for each individual cow when only subsequent calving date was available.

4.3.7. Statistical analyses

Latent class model

The estimated cow-level PT was then found using Bayesian latent class analysis using a similar model structure to that proposed by Branscum et al. (2005). For the k-th herd, the nk observations give the data vector for the joint test results y11, y10, y01, y00. Where y11 is the number of animals from the kth

herd that test positive on test 1 and test 2 and y10 is the number that test positive on test 1 and negative on test 2 and so on. y1 to yk are assumed to have multinomial sampling distributions:

$yk \sim multinomial(nk, (p11k, p10k, p01k, p00k))$

The multinomial cell probabilities are given by:

$p11k = \pi k \times Se1 \times Se2 + (1 - \pi k) \times (1 - Sp1) \times (1-Sp2)$

$p10k = \pi k \times Se1 \times (1-Se2) + (1 - \pi k) \times (1 - Sp1) \times Sp2$

$p01k = \pi k \times (1-Se1) \times Se2 + (1 - \pi k) \times Sp1 \times (1-Sp2)$

$p00k = \pi k \times (1-Se1) \times (1-Se2) + (1 - \pi k) \times Sp1 \times Sp2$

Where πk is the prevalence in the k-th herd and Se1, Se2 and Sp1, Sp2 are the sensitivities and specificities of test 1 and test 2 respectively. We modelled the prevalence in the k-th herd using a logit-normal distribution:

$Logit(\pi k) \sim \alpha + \psi k$

Where α is the logit of the prevalence of the k+1 herd, modelled as a normal distribution and ψ is the variation in prevalence for the k-th herd:

$\alpha \sim normal(\mu, 1/\sigma 2)$

$\psi k \sim normal(0, 1/\tau)$

$\tau \sim gamma(s1, s2)$

Sensitivity and specificity of each test were modelled using beta distributions.

Se1,2 ~ beta (alpha Se1,2, beta Se1,2)

Sp1,2 ~ beta (alpha Sp1,2, beta Sp1,2)

The model assumed conditional independence between tests. As a result, associations between tests are only explained by the latent variable (IAI prevalence in the population and in herds). Posterior inferences for each parameter were obtained using the R statistical software (R Core Team, 2015) (package 'rjags'; Plummer et al., 2019) using Markov chains, ran for 15,000 iterations after a burn-in period of 5,000 iterations. Convergence of the Markov chains was assessed by visual assessment of Markov chain and autocorrelation plots and by running multiple (n = 2) chains from distinct starting values (e.g., 0.05 and 0.95 for variables bounded between 0 and 1).

4.3.8. Model priors

4.3.8.1. Prevalence

A literature review was conducted to establish priors for the prevalence of IAI used in the BLCM. For this review, keywords used for the search were "dairy cow" AND "artificial insemination" AND "inaccurate" as words in the titles or the abstracts and searching was conducted in both the CABI direct database and Google Scholar search engine. However, reference lists of selected papers were also screened for further related research. The inclusion information required were as follows; 1.) a test had to be conducted at the time of AI to determine it as inaccurate, 2.) details of the study population (i.e., country, and dairy management system) had to be stated and 3.) the prevalence (or proportion) of IAI and the total number of animals sampled had to be stated. Additional information that was of interest were the types of tests, thresholds used to determine positivity, biological samples tested, and any further relevant detail. Initial screening was conducted on the title and abstract and non-relevant texts were immediately omitted. The second stage was conducted by screening the full manuscript. 25 research papers met this criterion and are summarised in Table 4.1. Only 6 research studies of the 25 matched our study population as they either took place on the island of Ireland or in countries with seasonal calving systems and as such were deemed appropriate. The mean prevalence of these studies was approximately 10% and these values assisted in selection for the prior prevalence estimate (mean = 10%, 95th percentile = 20%; Table 4.2). The corresponding alpha distribution was selected as normal (-2.4, 0.4) and is illustrated in Table 4.2. A gamma distribution of (10,10) was used as the prior for tau. The gamma distribution equates to the variance of the logit of normal distribution and considering our priors used, this allowed for the within herd prevalence to vary from anywhere from 0-100%.

Table 4.1 List of references used obtain the prior estimates of cow level prevalence of inaccurate artificial inseminations (IAIs). The reference, country, test used, sample type, threshold selected, cow level prevalence of AIs and the total number sampled in each study are summarised. Those studies with similar study populations were used to estimate the prior prevalence.

Studies with similar populations to present study population

Reference	Country	Test & Sample Type	Threshold for positivity stated	Prevalence of inaccurate AIs (total sampled)
McCaughey and Cooper, 1980	Northern Ireland	RIA & Defatted milk	≥ 0.3 ng/ml	7.7% (1177)
Fagan and Roche, 1986	Ireland	ELISA & Milk [b]	≥ 5 ng/ml	6% (309)
Cavestany and Galina, 2001	Uruguay	RIA & Defatted milk	≥ 1 ng/ml	11.1% (1215)
White and Sheldon, 2001	Northern Ireland	ELISA & Foremilk	≥ 5 ng/ml	19.7% (198)
McCoy et al., 2006	Northern Ireland	ELISA & Foremilk	≥ 2 ng/ml	12.3% (349)
Morton and Wynn., 2010	Australia	RIA & Foremilk	≥ 5 ng/ml	3.4% (6448 [a])
	Mean prevalence %			10%
	Weighted mean prevalence %			5.6% (9696)

Studies with different populations to present study population

Reference	Country	Test & Sample Type	Threshold for positivity stated	Prevalence of inaccurate AIs (total sampled)
Appleyard and Cook, 1976	UK	RIA & Blood	Repeated samples day 0, 2 and 4	21% (141)
Shemesh et al., 1978	Israel	RIA & Defatted milk	Not stated	16% (55)
Oltner and Edqvist, 1981	Sweden	RIA & Defatted milk	≥ 0.5 ng/ml	4% (1038) 12.2% (513) 31.2% (532)
Gowan et al., 1982	Canada	RIA & Milk [b]	≥1 ng/ml	11.7% (3014)
Ducker et al., 1983	UK	RIA & Milk [b]	≥1 ng/ml	14% (198)
Pennington et al., 1985	USA	RIA & Milk [b]	≥ 3 ng/ml	3.3 % (960)
Laitinen et al., 1985	Finland	RIA & Defatted milk & Milk [b]	Thresholds varied depending on sampled tested	4.6% (10000)

Table 4.1 Footnotes

[a]An approximation of the total number sampled based on total mean numbers of samples per herd x the number of seasonal herd enrolled (n = 124).

[b]Refers to whole milk.

Table 4.1 Continued on overleaf

Reference (Table 4.1 Continued)	Country	Test & Sample Type	Threshold for positivity stated	Prevalence of inaccurate AIs (total sampled)
Reimers et al., 1985	USA	RIA & Defatted milk	≥1 ng/ml	5.1 % (4558)
Nebel et al., 1987	USA	ELISA or RIA & Postmilk	≥ 5 ng/ml	18.7% (497) RIA 25.4% (497) ELISA
Senger et al. 1988	USA	RIA & Milk [b]	≥ 3 ng/ml	13% (4178)
Rajamahendran et al., 1990	Canada	ELISA or RIA & Foremilk	≥ 2 ng/ml	1.2% (256) RIA 3.9% (256) ELISA
Rajamahendran et al., 1993	Canada	RIA & Foremilk	≥ 1 ng/ml	4.8% (667)
Royal et al., 2000	UK	ELISA & Milk [b]	≥ 3 ng/ml	6% (682)
Sturman et al., 2000	USA	RIA & Postmilk	≥ 10 ng/ml	14.5% (380)
Kafi et al., 2007	Iran	RIA & Foremilk	≥ 3 ng/ml	10.7% (176)
Morton and Wynn, 2010	Australia	RIA & Foremilk	≥ 5 ng/ml	4.3% (2184 [a])
Ingenhoff et al., 2016	Australia	ELISA & Foremilk	≥ 5 ng/ml	3% (733)
Nyman et al., 2018	Sweden	RIA or ELISA & Milk [b]	Thresholds varied depending on assay	3.9% (2130)
LeRoy et al 2018	USA	ELISA & Blood	≥ 1 ng/ml	1.6% (919)
	Mean prevalence %			8.3%
	Weighted mean prevalence %			9.5% (32892)

4.3.8.2. Sensitivity and specificity

Although most of the studies estimating the prevalence of IAI used MP4, only a handful of authors stated test Se and Sp (Morton 2004; Morton and Wynn 2010; Ingenhoff et al., 2016) and in both of these studies, the target condition and/or Se and Sp definitions differed from the present study. Consequently, prior information about MP4 test was obtained from expert opinion, provided by two of the authors, MB and MC, both of whom have extensive research in the physiology of bovine estrous cycle (Forde et al. 2011). MP4 test Se estimate was decided on based on the authors' opinion that a cow that is presented for AI but not in estrus was quite likely to have an elevated MP4 (i.e., be in the luteal phase or pregnant), however, there are instances where the MP4 may be below the threshold and a cow may not be in estrus as she may be prior to or post estrus (so called 'periestrus' period) or in anestrus. To account for this, the authors made the following assumptions; 1.) worst case scenario, in any random group of cyclic cows, a progesterone concentration of above the threshold would be present at least two-thirds (14 days out of 21 days or 66% of the cycle) of the total estrous cycle (Lamming and Darwash, 1998), 2.) that if a pregnant cow was inseminated that it would be highly probable (>90%) that elevated progesterone would be detected (Pennington et al., 1985) and 3.) it would be highly improbable (<10% probability) that an anestrus cow would be inseminated even if prevalence of anestrus is reported to be between 13-48% by mating start date in seasonal systems (Rhodes et al., 2003). Consequently, an Se estimate was chosen (mode = 85%, 5th percentile = 65%; Table 4.2). In the case of MP4 test, Sp estimate was chosen (mode = 97%, 5th percentile = 90%; Table 4.2) on the basis that a cow that is presented for AI and in estrus was very likely to have a low MP4, because it is rare that a cow truly in estrus would have an elevated MP4 (Dobson et al., 1975).The corresponding Beta distribution for test Se and Sp were beta (15.13, 3.49) and beta (53.58, 2.63), respectively and are illustrated in Table 4.2.

Non-informative prior distributions were used for the Se and Sp of AC as no information has been published in the literature about its accuracy for determining IAI and they were deemed difficult to estimate by expert opinion. All prior distributions used in the BLCM are illustrated in Table 4.2.

Table 4.2. Estimates of the posterior distributions of the prevalence of inaccurate AIs at cow level. Estimates were used for prior probability distributions in the Bayesian Latent Class model.

	Estimate (Mode)	Limits of the estimate	Parameters
Cow Prevalence of inaccurate AI	0.10 [e]	0.20 [g]	normal (-2.4,0.4)
Test 1 Sensitivity [a]	0.85 [f]	0.65 [h]	beta (15.13, 3.49)
Test 1 Specificity [b]	0.97 [f]	0.90 [h]	beta (53.58, 2.63)
Test 2 Sensitivity [c]	0.50	0.00	beta (1, 1)
Test 2 Specificity [d]	0.50	0.00	beta (1, 1)
Tau (τ)	100 [e]	157 [g]	gamma (10,10)

Table 4.2 Footnotes

[a]Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (≥ 5ng/ml).

[b]Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (< 5ng/ml).

[c]Test 2 sensitivity was defined as the proportion of cows that were not in estrus at AI and did not conceive to the sample AI.

[d]Test 2 specificity was defined as the proportion of cows that were in estrus at AI and did conceive to sample AI.

[e]mean value

[f] based on an expert opinion of the co-authors (MB & MC)

[g]95th percentile

[h]5th percentile

4.3.9. Sensitivity analysis

As part of our sensitivity analysis, several measures were undertaken: altering priors, changing MP4 thresholds, sub-setting the data, testing the model with simulated data and running a one-test model using only MP4 test.

4.3.9.1. Altering priors

The final model was checked by changing the prior information of MP4 test Se and Sp to non-informative beta (1, 1) distribution while retaining our prior distribution for alpha (Table 4.3). The gamma prior distribution for tau was also varied from gamma (1, 1) to (0.1, 0.1). Reduction in these distributions allowed for less variation in the within herd prevalence estimates and this was conducted to assess how changes in this distribution influenced our cow-level PT estimates (Supplementary materials, Section 4.8, Table 4.8).

4.3.9.2 Altering milk progesterone thresholds for positivity

Various MP4 concentration thresholds (3, 4 and 5 ng/ml) were tested, while retaining the informed priors for alpha, MP4 test Se and Sp. This was conducted to assess how varying thresholds influenced the estimates of cow-level PT of IAIs (Table 4.4).

4.3.9.3 Sub-setting the data

While retaining the MP4 concentration of 5 ng/ml and informed priors for alpha, MP4 test Se and Sp, subsets of the data were run only using herds where greater than 5 samples per herd (n herds=39 & n=371 samples) had been collected or where cows that only had a subsequent calving recorded were retained (n herds=115 & n=491 samples). This assessed if there was marked variance in the estimates from the initial model (n herds=125 & n=576 samples).

4.3.9.4 Testing the model with simulated data

Using a method previously described (McAloon et al., 2019), we simulated PA data for both tests using the final model estimates and then re-applied the model to these simulated data to determine whether the initial parameter estimates could be recovered. This was conducted to assess the accuracy of our BLCA model in estimating model parameters, given previous observations that under certain disease and test characteristics, model estimates may be generated of low accuracy (McAloon et al., 2019).

4.3.9.5 Constructing one-test models using only milk progesterone

To check if our inference from the final two-test model was not diminished by using AC as a diagnostic test, we conducted a one-test model using MP4 test alone. To do this, we constructed three models; 1.) with the same Se and Sp priors of our two-test model; 2.) with Se and Sp priors based on Morton (2004)

and 3.) with Se and Sp priors based on Ingenhoff et al. (2016). The prior distribution for alpha remained constant in all three models with that of the two-test model.

4.4. Results

4.4.1. Descriptive Statistics

A total of 576 individual milk samples taken on the day of insemination were collected from 125 herds during the sampling period. The mean samples per farm was 4.7 with a range of 1-26 samples per farm. The average days in milk (DIM) at sampling was 90.7 DIM (SD 57.7; range 10-546). The average parity was 3.2 (range 1-11) and 94% (542/576) of cows calved in the spring months prior to sampling. The predominant breed sampled was Holstein-Friesian (83%;478/576) while the remaining were Holstein, Jersey, or Jersey crossbreds (17%; 98/576). The average predicted 305-day milk yield was 6,665KGS (SD 1291; range 3575-10725) and average Economic breeding Index value was 113€ (SD 60; range -98-247€).

The total number of milk samples with high progesterone (≥5 ng/ml) was 46 samples (7.9%). Of 576 milk samples, 452 samples were from first AI technician inseminations and of these, 29 samples (6.4%) had high progesterone. 124 samples were from repeat AI technician inseminations and of these 17 (13.7%) had high progesterone. The frequency distribution of MP4 concentrations in samples collected at the time of AI are illustrated in Figure 4.2.

Of the 576 cows, 491 had a subsequent calving registered of which 281 calved within a gestational range of 270-290 days (re-calving rate of approximately 48%). Of the 85 cows with no subsequent calving registered only 26 had departed each farm before 300 days after the sampled AI and thus may have been in calf and were removed for other reasons.

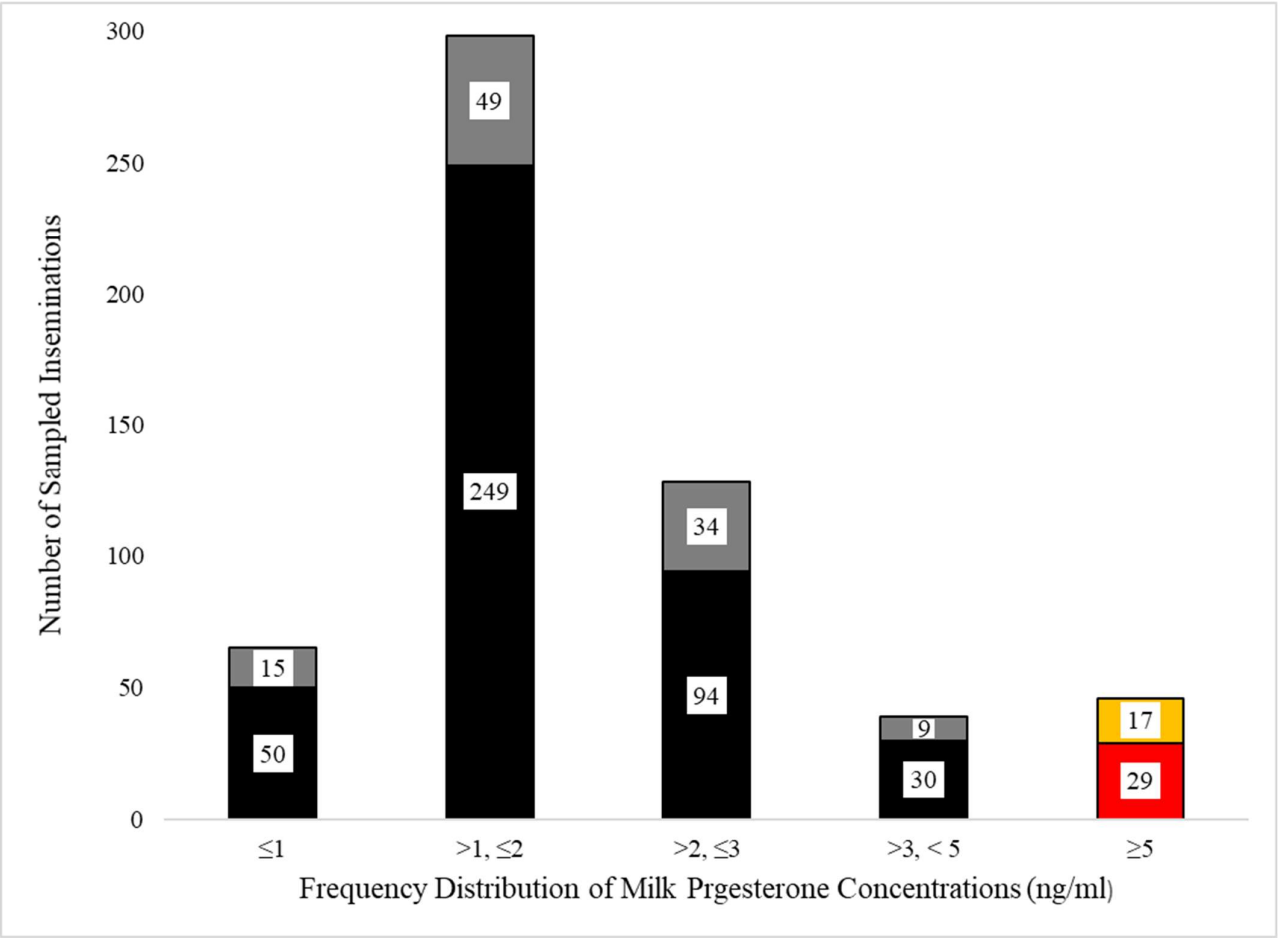


Figure 4.2. Frequency Distribution of the milk progesterone concentrations in samples collected at the time of AI (n=576). Grey (First Insemination samples) and Black (Repeat Insemination samples) bars indicate samples that were not classified as inaccurate; Red (First Insemination samples) and Yellow (Repeat Insemination samples) bars highlight samples that had were classified as inaccurat

4.4.2. Bayesian Latent Class Model Results

Final (informative) model

Cross-tabulated test results that were used in the final (informative) model are presented as supplementary materials (Section 4.8; Table 4.7). All results of the final model and non-informative are summarised in Table 4.3. A density plot of the cow-level and within herd PT of IAI is illustrated in Figure 4.3 (Plot A). Median (95% Bayesian Probability Interval; BPI) cow-level PT of IAI was 4.4% (BPI; 1.7–9.0%). Median Se and Sp estimates for MP4, were 83.0% (BPI; 65.0–96.2%) and were 97.4% (BPI; 94.6–99.6%), respectively. Median Se and Sp estimates for AC, were 64.8% (BPI; 44.5–88.6%) and 49.8% (BPI; 45.3–54.1%), respectively.

Table 4.3. Posterior probability estimates for the prevalence of inaccurate AIs at cow level. The median (mean) and Bayesian 95% probability interval (95% BPI) for the cow prevalence of inaccurate AI, sensitivity, and specificity for the latent class models with informative (final model) or noninformative priors.

	Final Model		**Noninformative model***	
	Median (Mean)	**95% BPI**	**Median (Mean)**	**95% BPI**
Cow Prevalence	0.044 (0.042)	0.017-0.090	0.055 (0.057)	0.013-0.193
Test 1 Sensitivity [a]	0.830 (0.818)	0.650-0.962	0.727 (0.692)	0.284-1.00
Test 1 Specificity [b]	0.974 (0.972)	0.946-0.996	0.977 (0.972)	0.937-1.00
Test 2 Sensitivity [c]	0.648 (0.653)	0.445-0.886	0.638 (0.644)	0.427-0.900
Test 2 Specificity [d]	0.498 (0.498)	0.453-0.541	0.500 (0.499)	0.449-0.554

Table 4.3 Footnotes.

[a] Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (≥ 5ng/ml).

[b] Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (< 5ng/ml).

[c] Test 2 sensitivity was defined as the proportion of cows that were not in estrus at AI and did not conceive to the sample AI.

[d] Test 2 specificity was defined as the proportion of cows that were in estrus at AI and did conceive to the sample AI.

* beta () for both tests was beta (1,1) while normal () for prevalence remained as normal (-2.4,0.4)

4.4.3 Sensitivity analysis

4.4.3.1. Results of altering priors

Results of the model using non-informative priors for MP4 for Se and Sp [beta(1.0, 1.0)] suggest no impact of prior information on the cow-level PT estimate of IAI with the median PT between both models relatively similar (median 4.4% versus 5.5%; Table 4.3). Median Se estimates for MP4 did alter between models with 72.7% versus 83.0% in the non-informative and informative models, respectively. There was little difference in median Sp estimates for MP4 between models with 97.7% versus 97.4% in the non-informative and informative models, respectively. The complete comparison between both models is illustrated in Table 4.3.

Reduction in the gamma prior distribution for tau resulted in a reduction in the median cow-level PT estimate of IAI. For a gamma (1, 1) distribution the median cow-level PT estimate of IAI was 3.0% (BPI; 0.8–8.1%) and for a gamma (0.1, 0.1) distribution the median estimate was 2.1% (BPI; 0.4–7.4%). There was little difference in the Se or Sp estimate of either test. The complete results are presented in the supplementary materials (Section 4.8, Table 4.8).

4.4.3.2. Results of altering milk progesterone thresholds for positivity

There was a moderate effect on the cow-level PT estimate of IAI (median; 4.4% to 6.3%; Table 4.4) by varying the MP4 threshold from ≥ 5ng/ml to ≥ 4ng/ml in the final model. However, there was a larger effect on the cow-level PT estimate of IAI (median; 4.4% to 10.9%; Table 4.4) by varying the MP4 threshold from ≥ 5ng/ml to ≥ 3ng/ml in the final model.

Table 4.4. The median (mean) and Bayesian 95% probability interval (95% BPI) for the cow level prevalence of inaccurate AI, sensitivity and specificity for the latent class models obtained by varying the thresholds of milk progesterone concentration.

Threshold	Probability posterior estimates	Median (Mean)	95% BPI
≥ 5ng/ml			
	Cow Prevalence	0.044 (0.042)	0.017-0.090
	Test 1 Sensitivity [a]	0.830 (0.818)	0.650-0.962
	Test 1 Specificity [b]	0.974 (0.972)	0.946-0.996
	Test 2 Sensitivity [c]	0.648 (0.653)	0.445-0.886
	Test 2 Specificity [d]	0.498 (0.498)	0.453-0.541
≥ 4ng/ml			
	Cow Prevalence	0.063 (0.062)	0.032-0.116
	Test 1 Sensitivity [a]	0.837 (0.826)	0.667-0.965
	Test 1 Specificity [b]	0.977 (0.975)	0.950-0.998
	Test 2 Sensitivity [c]	0.597 (0.600)	0.437-0.773
	Test 2 Specificity [d]	0.497 (0.497)	0.453-0.497
≥ 3ng/ml			
	Cow Prevalence	0.109 (0.108)	0.063-0.180
	Test 1 Sensitivity [a]	0.837 (0.828)	0.671-0.964
	Test 1 Specificity [b]	0.975 (0.973)	0.944-0.997
	Test 2 Sensitivity [c]	0.583 (0.584)	0.460-0.706
	Test 2 Specificity [d]	0.500 (0.501)	0.454-0.547

Table 4.4 Footnotes

[a] Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (threshold concentration tested indicated in table).

[b] Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (threshold concentration tested indicated in table).

[c] Test 2 sensitivity was defined as the proportion of cows that were not in estrus at AI and did not conceive to the sampled AI.

[d] Test 2 specificity was defined as the proportion of cows that were in estrus at AI and did conceive to the sampled AI.

4.4.3.3. Results of sub-setting the data

Assessment of various subsets of the data, where only using data from herds were greater than 5 samples (n herds=39 & n=371 samples) had been collected or where cows with only subsequent calving were retained (n herds=115 & n=491 samples) showed marginal variance on the final BLCM (Table 4.5, overleaf).

Table 4.5. The mean (median) and Bayesian 95% probability interval (95% BPI) for the cow-level prevalence of inaccurate AI, sensitivity, and specificity for the Bayesian latent class models after sub-setting the data. Subset Model 1 = subset of the full data where only herds where greater than 5 samples (n herds=39 & n=371 samples) had been collected. Subset Model 2 = subset of the full data where cows with only subsequent calving were retained (n herds=115 & n=491 samples). Milk progesterone threshold remained at 5ng/ml and informed priors remained constant across models.

Model	Probability posterior estimates	Median (Mean)	95% BPI
Final Model			
	Cow Prevalence	0.044 (0.042)	0.017-0.090
	Test 1 Sensitivity [a]	0.830 (0.818)	0.650-0.962
	Test 1 Specificity [b]	0.974 (0.972)	0.946-0.996
	Test 2 Sensitivity [c]	0.648 (0.653)	0.445-0.886
	Test 2 Specificity [d]	0.498 (0.498)	0.453-0.541
Subset Model 1			
	Cow Prevalence	0.044 (0.043)	0.018-0.095
	Test 1 Sensitivity [a]	0.831 (0.820)	0.657-0.965
	Test 1 Specificity [b]	0.976 (0.975)	0.950-0.996
	Test 2 Sensitivity [c]	0.700 (0.699)	0.481-0.937
	Test 2 Specificity [d]	0.526 (0.526)	0.471-0.579
Subset Model 2			
	Cow Prevalence	0.037 (0.033)	0.009-0.093
	Test 1 Sensitivity [a]	0.822 (0.811)	0.642-0.966
	Test 1 Specificity [b]	0.968 (0.966)	0.934-0.995
	Test 2 Sensitivity [c]	0.547 (0.555)	0.268-0.881
	Test 2 Specificity [d]	0.579 (0.579)	0.532-0.624

Table 4.5 Footnotes

[a] Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (threshold concentration was >5ng/ml).

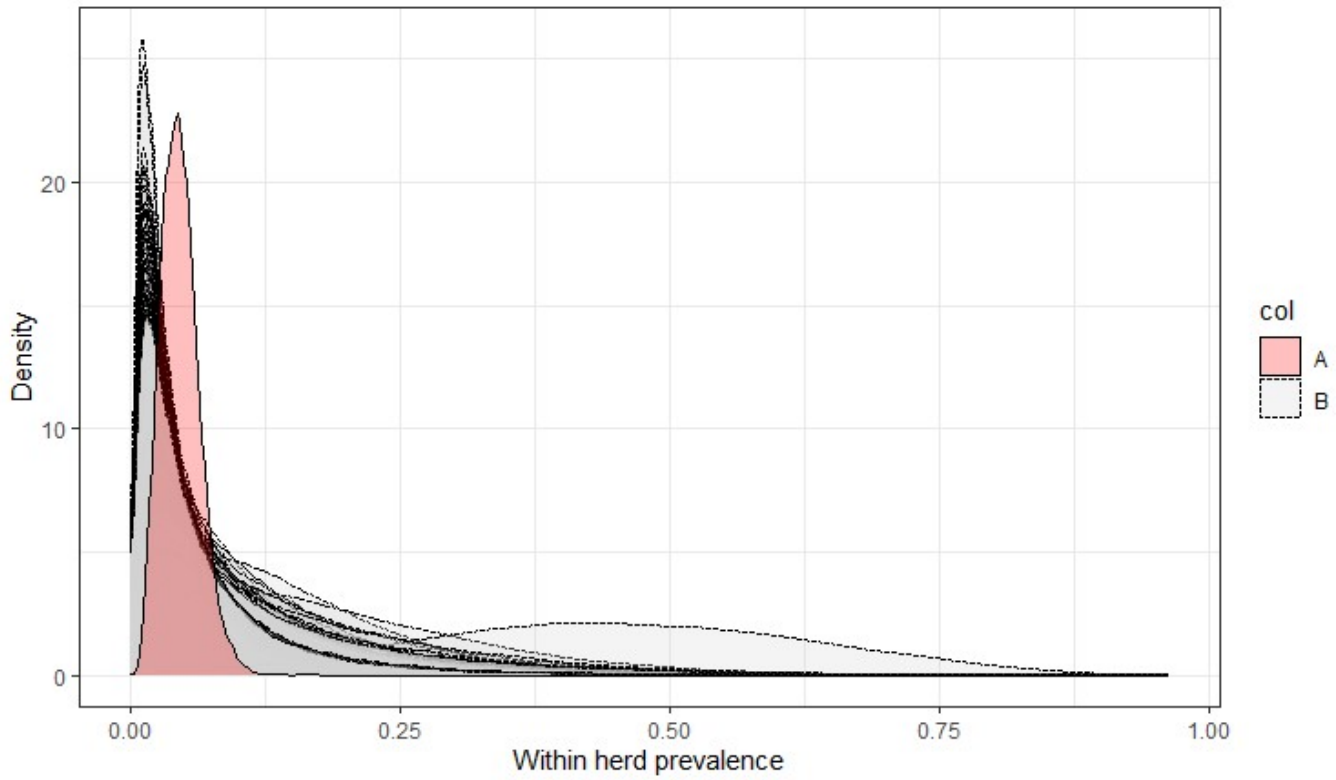
[b] Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (threshold concentration was >5ng/ml).

[c] Test 2 sensitivity was defined as the proportion of cows that were not in estrus at AI and did not conceive to the sampled AI.

[d] Test 2 specificity was defined as the proportion of cows that were in estrus at AI and did conceive to the sampled AI.

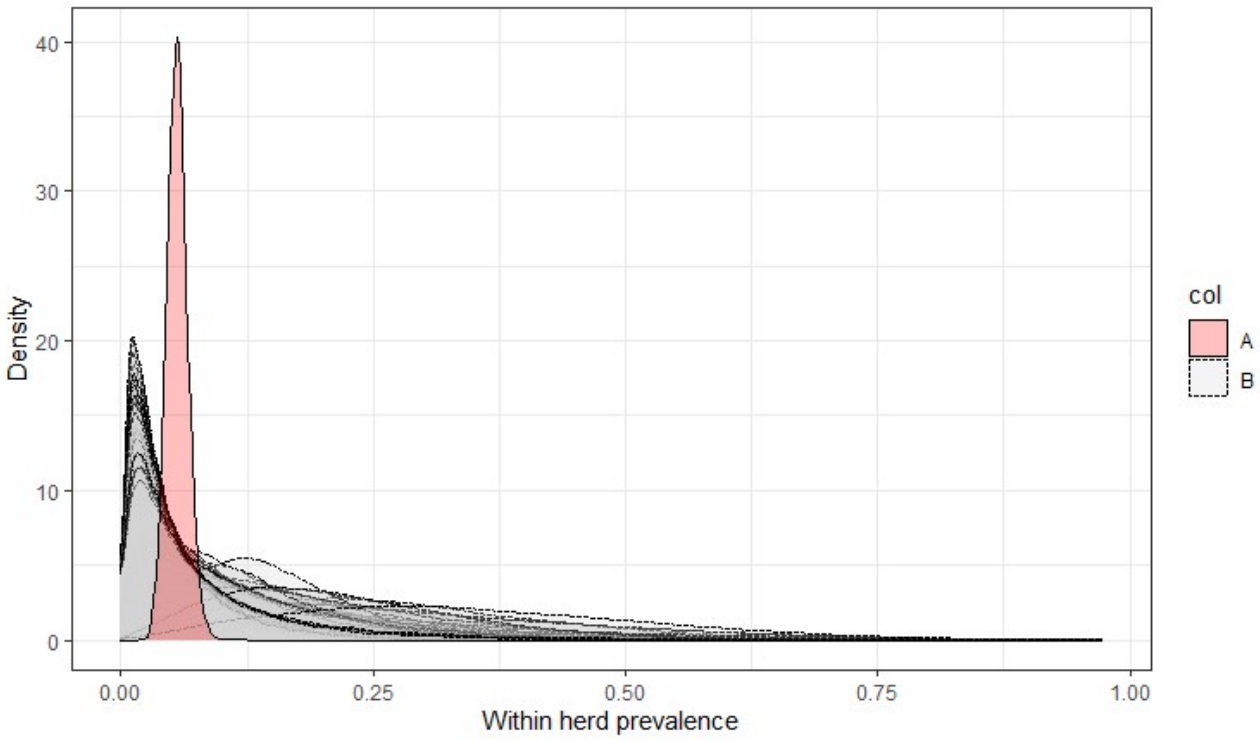
4.4.3.4. Results of testing the model with simulated data

The estimates from the simulated data (median estimate of 5.2%) were similar that of the final model (median estimate of 4.4%) indicating that the BLCA was accurate in its predictions of cow-level PT estimate of IAI. The density plots of the of the cow-level and within herd PT of IAI for the sampled and simulated data are presented in Figure 4.3(Plot A & B) for comparative purposes.



A. Data Plot

Figure 4.3 A. Density (y-axis) plots of cow level true prevalence (A; red shaded solid line) and the within-herd prevalence (B; grey shaded, dotted lines). Plot A (Data Plot) illustrates these posterior estimates based on the sampled data from the final Bayesian latent class model. By comparing both plots (A & B overleaf) it is noted that the cow level true prevalence estimates are similar, although the within-herd prevalence estimates vary.



B. Simulated Plot

Figure 4.3 B. Density (y-axis) plots of cow level true prevalence (A; red shaded solid line) and the within-herd prevalence (B; grey shaded, dotted lines). Plot B (Simulated Plot) was constructed as part of the sensitivity analysis by simulating synthetic new data based using the estimated parameters from the final model and then re-applying them in the same final model to test if these estimates could be recovered. By comparing both plots (A & B overleaf) it is noted that the cow level true prevalence estimates are similar, although the within-herd prevalence estimates vary.

4.4.3.5. Results of one-test models using only milk progesterone

The results of the one-test model using MP4 test alone, the same Se and Sp priors based our two-test model and the work of other authors (Morton 2004; Ingenhoff et al., 2016) illustrated broadly similar cow-level PT estimates of IAI across all three models. Median cow-level PT estimates were 4.9% (BPI; 2.1-9.3%), 2.4% (BPI 0.8-6.0%) and 4.6% (BPI; 2.3-8.2%) based on Se and Sp priors from the present study's two test model, Morton (2004) and Ingenhoff et al. (2016), respectively. Sp estimates were comparable in all three models (97.9%, 94.9% and 98.2%, respectively) but Se was lower in the case of our one-test model based on our two test priors (83.4%) versus the one-test models that we based on Se and Sp priors taken from the literature (95.5% and 89.5%). The complete results are presented in Table 4.6 (overleaf).

Table 4.6 The mean (median) and Bayesian 95% probability interval (95% BPI) for the cow level prevalence of inaccurate AI (IAI), sensitivity and specificity for a one-test latent class model. Final Priors = Se and Sp priors used in the two-test model based on expert opinion of the co-authors were used. Alternative Priors 1 = Se and Sp priors based on the work of Morton (2004) [(Se = 0.97 (95% CI 0.88-1.00) ; Sp = 0.86 (95% CI 0.75-0.94)]. Alternative Priors 2 = Se and Sp priors based on the work of Ingenhoff et al. (2016) [(Se = 90.1% (95% CI 0.836–0.966) ; Sp = 98.7% (95% CI 0.961–1.00)]. Milk progesterone threshold remained at 5ng/ml and informed priors for alpha remained constant across models.

Model	Probability posterior estimates	Median (Mean)	95% BPI
Final Priors			
	Cow Prevalence	0.049 (0.047)	0.021-0.093
	Test 1 Sensitivity [a]	0.834 (0.822)	0.657-0.966
	Test 1 Specificity [b]	0.979 (0.977)	0.953-0.998
Alternative Priors 1			
	Cow Prevalence	0.024 (0.022)	0.08-0.06
	Test 1 Sensitivity [a]	0.955 (0.947)	0.881-0.997
	Test 1 Specificity [b]	0.949 (0.948)	0.919-0.977
Alternative Priors 2			
	Cow Prevalence	0.046 (0.045)	0.023-0.082
	Test 1 Sensitivity [a]	0.895 (0.892)	0.826-0.954
	Test 1 Specificity [b]	0.982 (0.980)	0.960-0.998

Table 4.6 Footnotes

[a] Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (threshold concentration was >5ng/ml).

[b] Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (threshold concentration was >5ng/ml).

4.5. Discussion

The present study estimated the cow-level PT of IAI using a hierarchical Bayesian latent class model which assumed conditional independence between tests. This is an important assumption as failure to do so may led to potential bias in parameter estimation (Albert and Dodd, 2004).

To the authors knowledge, this is the first time this type of approach has been used to estimate the cow-level PT of IAI in the literature and illustrates a novel approach for doing so. In veterinary epidemiology, Bayesian latent class analysis has been used extensively to approximate herd and animal-level PT of disease (Branscum et al., 2004), however, this approach illustrates that it has applications in veterinary research beyond the study of disease and in other areas such as, reproductive management.

IAIs are costly in terms of wasted semen but even more so in terms of the technician time and labour (Sturman et al., 2000). This is probably even more profound in seasonal breeding systems, such as Ireland, where the demand on technician's time and effort is great due to the condensed nature of artificial breeding. Therefore, estimation of the current cow-level PT of IAIs performed in the present study was not only important to inform breeding companies as to the number of AIs that are wasted but also to give some perspective on the current accuracy of estrus detection on Irish dairy farms. This will in turn assist in further technician and farmer training around the signs of estrus resulting in more efficient semen use and less potential iatrogenic abortion in pregnant cows (Sturman et al., 2000). In the present study, the final model estimated cow-level PT of IAI in Irish milk recording dairy herds to have a median of 4.4% (BPI; 1.7-9.0%) which is generally promising as it illustrates relatively low rates of semen wastage, and that good estrus detection accuracy is likely on most Irish dairy farms.

Unfortunately for comparative purposes, there are only handful of reports that have given some idea of the prevalence of IAI in Irish dairy herds and many of these are dated. Over 30 years ago, Fagan and Roche (1986) reported that 6% (19/309) of AIs in a sample of 5 herds in the Republic of Ireland were inaccurate. While other studies in Northern Ireland, have estimated 7.7% (McCaughey and Cooper, 1980), 19.7% (White and Sheldon, 2001) and 12.3% (McCoy et al., 2006) of all sampled AIs were inaccurate. Temporal trends as well as the unique differences in the study population of dairy cows in Northern Ireland, where there is a greater tendency for intensive indoor systems, might explain why our estimate was lower than previous work (White and Sheldon 2001; McCoy et al., 2006). Probably the cow-level PT estimate that most closely resembled the present study was that noted by Morton and Wynn (2010) in a study involving 124 seasonal calving herds in Australia and similar recent work conducted by this research group (see Chapter 5). Also using a 5ng/ml threshold of MP4, the mean estimates found in these studies was 3.4% (Morton and Wynn 2010) and 4.7% (Kelly et al., 2021; Chapter 5) which is similar to the present study's estimates. However, comparisons with any of these studies are complicated further by the fact that they were all PA and not adjusted for test Se and Sp. Additionally, biological samples tested, laboratory methods of progesterone determination and the MP4

threshold concentration were not consistent between all studies (Table 4.1). The effect of altering threshold is noteworthy, as during the sensitivity analysis when the threshold of MP4 was altered, the PT estimate resembled or was higher than earlier Irish PA estimates of 6% and 7.7% when a threshold concentration of 4ng/ml (median: 6.3%, BPI; 3.2-11.6%) or 3ng/ml (median: 10.9%, BPI; 6.3-18.0%) was selected.

In the final model, MP4 test Se and Sp were estimated at a mean of 83.0% (BPI; 65.0-96.2%) and 97.4% (BPI; 94.6-99.6%), respectively. Median Se estimates for the MP4 test did differ between non-informative and informative models (72.7% versus 83.0%) but there was little difference in test Sp (97.7% versus 97.4%). This may indicate that MP4 test Se was indeed lower than our prior estimates selected by our experts, although this difference had minimal effect on estimated cow-level PT . Of the few other studies that reported Se and Sp for various MP4 tests, the results are comparable, although our MP4 test Se estimate appears lower than most. Ingenhoff et al., (2016) estimated a Se and Sp for a MP4 ELISA as 90.1% (95% CI 0.836–0.966) and 98.7% (95% CI 0.961–1.00) which are both higher than the results of the present study, however, the MP4 test (i.e., an ELISA) and the test Se and Sp definitions differed between that study and the present. In that same study (Ingenhoff et al., 2016), the target condition was the presence of a corpus luteum (CL) and this was detected by ultrasonography, a test which in itself does not have perfect Se (91.4–96.7%) (Lean et al., 1992). As a result of this inclusion criteria, test Se was defined as the percentage of CL-positive cows that had a high progesterone result while Sp defined as the percentage was the percentage of CL-negative cows that had a low progesterone result. In the case of the present study, although our target condition was cows not in estrus and that this condition would include cows with a CL (i.e., either cows in the luteal phase or pregnant), it would also include cows in periestrus and anestrus so it would not be expected that test Se would be as high. In a study by Morton and Wynn (2010) that used a similar MP4 test (i.e., RIA) and a similar threshold (≥ 5ng/ml), test Se and Sp were both estimated at greater than 85%. However, in that study, Se was defined as the proportion of cows in the luteal phase that are classified as having ‘high’ progesterone and test Sp as the proportion of ovulating cows that are classified as not having ‘high’ progesterone (Morton 2004). As the present study had a broader target condition (i.e, cows not in estrus) and defined test Se as the proportion of cows that were not in estrus at AI, it is not surprising that our estimate for MP4 test Se were slightly lower than previously published work (Morton and Wynn 2010; Ingenhoff et al., 2016). Incidentally, this may also explain why during the sensitivity analysis, the median estimate for cow-level PT of IAI was lower in the one test model (2.4% versus 4.4%) when Se and Sp priors were based on Morton’s research (2004) versus that of the present study. Our estimate for test Sp was high (~97%) and similar to our prior estimate in our non-informative model and other published research (Morton and Wynn 2010; Ingenhoff et al. 2016) indicating that very few cows in estrus at AI would be detected as having elevated MP4.

In the final model, AC test Se and Sp were estimated at a median of 64.8% (BPI; 44.5-88.6%) and 49.8% (BPI; 45.3-54.1%), respectively. Test Se in this case indicates that on average, approximately two thirds of animals who are not in estrus at AI will calve outside of a normal gestational interval. This is probably not surprising considering they may be missed at a subsequent estrus if inseminated incorrectly in the luteal phase or in anestrus or that many may already be pregnant when inseminated (Sturman et al., 2000). The remaining cows that were not in estrus at the sampled AI but do calve within a normal gestational interval are likely cows that are inseminated at a repeat estrus event occurring at a short interval after the AI (i.e., 3-17 days later). Test Sp for the AC test was poor (approximately 50%). Again, this finding was not surprising as it was close to the observed re-calving rate seen in typical Irish dairy cows (Diskin et al., 2011). This can be explained as follows, if a seasonally calving moderate yielding dairy cow who is in estrus is inseminated at the correct time, a fertilization rate of 90-95% would be expected and if there is approximately a 40% embryonic and fetal mortality rate, 50-55% of cows should re-calve to a particular AI (Diskin et al., 2011). Therefore, as test Sp for the AC test was defined as those cows in estrus that did conceive and re-calve within a normal gestational period, it is not surprising that test Sp closely resembles that of the re-calving rate.

The AC test used in this study has some limitations: it is retrospective in its determination and has the potential to be impacted by cows that do conceive at an AI relatively close to a sampled AI or by cows that do conceive but do not re-calve for reasons unrelated to the AI being inaccurate (e.g., involuntary culling for lameness, mastitis, age, abortion etc.). Additionally, this test is further limited, as the BLCM assumes that Se and Sp of all tests are constant across herds which may not necessarily be the case of the AC test as re-calving rates may vary between herds for reasons outlined above. However, the purpose of its inclusion in the BLCM was to inform the cow-level PT of IAI. The Se or Sp of this diagnostic 'test' were not of interest. Subsets of the dataset were also modelled during the sensitivity analysis, to include only cows that had subsequent calving dates recorded (n=491 samples in 115 herds; Table 4.5) and cow-level PT estimates, test Se or Sp did not differ considerably indicating inclusion of these cows did not bias the estimates. Lastly, the one-test models that were constructed using only the MP4 test resulted in similar cow-level PT estimates of IAI indicating that the AC test was not negatively impacting the quality of our inference.

One of the limitations of the present study was that the sampling strategy did not facilitate an equal number of AIs to be selected per herd and it restricted sampling to only herds that were using a breeding company for AI. Due to the study design, it was only possible for AIs that took place on the same day as their routine milk recording to be sampled which meant that even in seasonal herds, small herd size may have limited number of cows in estrus each day and they will also suffer from temporal shift. For example, in a 100-cow seasonal herd, it would be expected that 4-5 cows be in estrus on any random day in the first three weeks, but this may diminish to 2-3 in the subsequent three weeks and so on. Thus, gathering a larger number of samples from larger herds and a smaller number of samples from smaller

herds has the potential to bias the estimate. Although calculating an accurate estimate of the within herd prevalence of was not the objective of this study, it was conducted as part of BLCM. In the present study maximum and minimum median estimated within herd prevalence were 2.2% and 44.9%, respectively and 21% (26/125) of herds had an estimated median prevalence of greater than 5%. Large variation in the within herd prevalence of IAI has been reported by some (Nebel et al., 1987; Cavestany and Galina 2001) while others have reported that a high (>5%) within herd prevalence can be an issue in 25% of herds (Morton and Wynn, 2010).

A further limitation of the sampling strategy was that only technician AIs were sampled. This means that no inferences can be made of the accuracy of estrus detection in Irish herds where so-called 'do-it-yourself' or farmer AI is routinely conducted. These sampling issues mean that although the overall cow-level PT of IAI appears relatively low, it does not diminish the fact that it may be a significant an issue in a proportion of herds and that it could potentially be higher in herds that perform their own AI. Additionally, hormonal methods such as synchronization of estrus and ovulation, which is not commonplace in Irish seasonal herds but may reduce IAI requires further investigation. Further research on better estimating the within herd prevalence across a broader population of Irish dairy cows with different AI management systems is required to better measure the PT of IAI and the herd level risk factors for its occurrence.

4.6 Conclusion

The cow level true prevalence of inaccurate AIs in Irish dairy cows was estimated using a Bayesian latent class model which is the first time this approach has been used to do so. In the final model, median cow-level true prevalence of IAI was 4.4% (BPI; 1.7–9.0%). Median Se and Sp estimates for MP4, were 83.0% (BPI; 65.0–96.2%) and 97.4% (BPI; 94.6–99.6%), respectively. Median Se and Sp estimates for AC, were 64.8% (BPI; 44.5–88.6%) and 49.8% (BPI; 45.3–54.1%), respectively. The present study estimates that the overall cow-level true prevalence of IAI in Irish dairy cows is relatively low. Further research is required to better estimate the variation in within herd prevalence and get a better representation of Irish dairy cows.

4.7 Acknowledgements

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4.8 Supplementary Materials

Table 4.7 Cross-tabulated results for milk progesterone (MP4+; thresholds of ≥ 5ng/ml is test positive) and apparent conception (AC -; Not conceiving to sampled AI is test positive) used in the final model to estimate the prevalence of inaccurate AIs in 576 dairy cows from 125 Irish dairy herds.

Herd	MP4+ AC-	MP4 + AC+	MP4 - AC-	MP4 – AC +	Total
1	0	1	11	14	26
2	0	0	12	12	24
3	0	0	6	14	20
4	0	1	9	5	15
5	1	1	6	6	14
6	3	0	4	6	13
7	0	0	3	9	12
8	1	0	1	10	12
9	0	0	8	4	12
10	0	1	6	4	11
11	0	0	9	2	11
12	2	0	4	4	10
13	0	0	7	3	10
14	1	1	4	4	10
15	0	0	4	5	9
16	0	0	2	6	8
17	0	0	3	5	8
18	0	0	2	6	8
19	2	1	3	2	8
20	1	0	5	2	8
21	0	0	6	2	8
22	5	1	2	0	8
23	0	1	5	2	8
24	0	0	2	6	8
25	0	0	2	5	7
26	0	1	1	5	7
27	0	0	2	5	7
28	0	0	1	5	6
29	0	0	2	4	6
30	0	0	5	1	6
31	0	0	3	3	6
32	1	1	2	2	6
33	0	0	5	1	6

34	0	0	4	2	6
35	0	0	3	3	6
36	0	0	2	4	6
37	1	1	0	3	5
38	0	0	4	1	5
39	0	0	4	1	5
40	0	0	2	2	4
41	0	0	3	1	4
42	0	0	3	1	4
43	0	0	4	0	4
44	0	0	1	3	4
45	0	0	2	2	4
46	0	1	3	0	4
47	0	2	1	1	4
48	0	1	1	2	4
49	0	0	1	3	4
50	0	0	3	1	4
51	0	0	3	1	4
52	0	0	3	1	4
53	1	0	2	1	4
54	0	0	2	2	4
55	0	0	2	2	4
56	0	0	1	3	4
57	0	1	2	1	4
58	0	0	2	2	4
59	1	0	0	3	4
60	0	0	3	0	3
61	0	1	0	2	3
62	0	0	2	1	3
63	0	0	1	2	3
64	0	0	2	1	3
65	0	0	0	3	3
66	0	0	1	2	3
67	1	0	1	1	3
68	0	0	0	3	3
69	0	0	3	0	3
70	0	0	3	0	3
71	1	0	1	1	3
72	0	0	3	0	3
73	0	0	2	1	3

74	0	0	1	2	3
75	1	0	0	2	3
76	0	0	1	2	3
77	0	0	2	1	3
78	0	0	1	2	3
79	0	0	1	1	2
80	0	0	0	2	2
81	0	0	1	1	2
82	0	0	2	0	2
83	1	0	0	1	2
84	0	0	1	1	2
85	0	0	2	0	2
86	0	0	1	1	2
87	0	0	0	2	2
88	0	0	1	1	2
89	0	0	1	1	2
90	0	0	0	2	2
91	0	0	2	0	2
92	1	0	1	0	2
93	0	0	1	1	2
94	0	0	2	0	2
95	0	0	0	2	2
96	0	0	2	0	2
97	0	0	1	1	2
98	0	0	2	0	2
99	0	0	1	1	2
100	0	0	1	0	1
101	1	0	0	0	1
102	0	0	1	0	1
103	1	0	0	0	1
104	0	0	1	0	1
105	0	0	1	0	1
106	0	1	0	0	1
107	0	1	0	0	1
108	0	0	0	1	1
109	0	0	1	0	1
110	0	0	0	1	1
111	0	0	1	0	1
112	0	0	0	1	1
113	0	0	0	1	1

114	0	0	1	0	1
115	0	0	1	0	1
116	0	0	0	1	1
117	0	0	1	0	1
118	0	0	0	1	1
119	0	0	1	0	1
120	0	0	0	1	1
121	0	0	1	0	1
122	0	0	1	0	1
123	0	0	0	1	1
124	0	0	1	0	1
125	0	0	1	0	1
Grand Total	27	19	268	262	576

Table 4.8. The mean (median) and Bayesian 95% probability interval (95% BPI) for the cow level prevalence of inaccurate AI, sensitivity and specificity for the latent class models obtained by varying the gamma prior distribution for tau. Milk progesterone threshold remained at 5ng/ml and informed priors remained constant across models.

Gamma Distribution	Probability posterior estimates	Median (Mean)	95% BPI
Final Model (10, 10)			
	Cow Prevalence	0.044 (0.042)	0.017-0.090
	Test 1 Sensitivity [a]	0.830 (0.818)	0.650-0.962
	Test 1 Specificity [b]	0.974 (0.972)	0.946-0.996
	Test 2 Sensitivity [c]	0.648 (0.653)	0.445-0.886
	Test 2 Specificity [d]	0.498 (0.498)	0.453-0.541
(1, 1)			
	Cow Prevalence	0.03 (0.028)	0.008-0.081
	Test 1 Sensitivity [a]	0.824 (0.813)	0.645-0.962
	Test 1 Specificity [b]	0.971 (0.970)	0.943-0.994
	Test 2 Sensitivity [c]	0.664 (0.668)	0.454-0.894
	Test 2 Specificity [d]	0.499 (0.499)	0.455-0.542
(0.1, 0.1)			
	Cow Prevalence	0. 021 (0.019)	0.04-0.074
	Test 1 Sensitivity [a]	0.818 (0.808)	0.634-0.960
	Test 1 Specificity [b]	0.968 (0.967)	0.941-0.993
	Test 2 Sensitivity [c]	0.675 (0.678)	0.456-0.903
	Test 2 Specificity [d]	0. 499 (0.499)	0.456-0.542

[a]Test 1 sensitivity was defined as the proportion of cows that were not in estrus at AI and detected with elevated progesterone (threshold concentration was >5ng/ml).

[b]Test 1 specificity was defined as the proportion of cows that were in estrus at AI and detected with low progesterone (threshold concentration was >5ng/ml).

[c]Test 2 sensitivity was defined as the proportion of cows that were not in estrus at AI and did not conceive to the sampled AI.

[d]Test 2 specificity was defined as the proportion of cows that were in estrus at AI and did conceive to the sampled AI.

Chapter 5

Cow-level prevalence and risk factors for estrus detection inaccuracy in seasonal calving pasture-based dairy cows.

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5.0. Abbreviations used in this chapter

AI: artificial insemination

AOR: Adjusted odds ratio

EDI: Estrus detection inaccuracy [Please note for the purposes of this thesis this is synonymous with Inaccurate artificial insemination (IAI) described in chapter 4]

ELISA: Enzyme-linked immunosorbent assay

IQC: Internal quality control

MP4: Milk progesterone

MSD: Mating start date

OR: Odds ratio

P4: Progesterone

PTA: Predicted transmitting ability

RIA: Radioimmunoassays

SD: Standard Deviation

5.1 Abstract

High submission rates and pregnancies per artificial insemination (**AI**) are essential to ensure compact calving is achieved in seasonal calving pasture-based systems. Estrus detection inaccuracy (**EDI**) is one area that negatively impacts pregnancies per AI as it increases the inseminations per pregnancy with little probability of conception, while also having the potential to disrupt established pregnancies. The aims of this cross-sectional study were to provide cow-level estimates of EDI prevalence and determine cow-level risk factors for EDI in seasonal calving pasture-based systems. A total of 1071 milk samples were obtained from 984 cows on 19 farms in spring 2018 and analyzed by radioimmunoassay to determine the progesterone concentration at the time of artificial insemination. Based on a validation study on a subset of cows, an inaccurate estrus detection was described as a concentration of progesterone in foremilk of ≥ 3 ng/ml which corresponded to a composite milk progesterone value of 5 ng/ml. To investigate selected risk factors for EDI, we conducted statistical analyses using two multivariate logistic regression models, stratifying by insemination number (first versus repeat). The overall prevalence of EDI was 4.7% with a prevalence of 3.3% of EDI at first insemination and 14.1% at repeat insemination. Absence of a mounting abrasion (Adjusted odds ratio (**AOR**) = 3.0) was a significant risk factor for EDI on first insemination while abnormal preceding repeat interval (AOR = 9.5), the absence of an observed standing estrus (AOR = 12.5) and the absence of a mounting abrasion (AOR = 4.1) were significant risk factors for EDI on repeat insemination. The results indicate that cow-level estimated prevalence of EDI in a selection of pasture-based herds was low at first insemination but higher for repeat insemination. It confirms that certain cow-level risk factors existed for EDI, thus providing preliminary evidence for potential future investigation into the targeted use of on-farm progesterone assays in pasture-based herds.

5.2 Introduction

Reproductive efficiency is essential to the profitability of seasonal calving pasture-based dairy herds (Veerkamp et al., 2002). Good reproductive management in pasture-based herds delivers a compact period of calving so dietary demand of peak lactation coincides with optimal grass growing conditions (Dillon et al., 1995). High submission and pregnancies per AI are essential to ensure compact calving is to be achieved in such pasture-based systems (McDougall et al., 2006). Factors that negatively influence submission rates, pregnancies per AI or both are well documented in seasonal herds and include; negative energy balance or excessive body condition (**BCS**) loss post-partum (Roche et al., 2007), genetic potential of the cow or herd (Carty et al., 2020), dispersed existing calving pattern (Lane et al., 2013) and poor estrus detection sensitivity or 'efficiency' (Morton and Wynn, 2010). Estrus detection inaccuracy (**EDI**) refers to the detection of estrus and subsequent insemination in a cow where ovulation is not pending (also termed 'false positive insemination' (Morton, 2004)). EDI is an economic loss to the dairy industry as it negatively impacts pregnancies per AI through increasing the inseminations per pregnancy with little probability of conception (Appleyard and Cook, 1976) and risking iatrogenic abortions in already pregnant cows (Sturman et al., 2000). Despite this, many of the economics and overall effects on herd or individual cow reproductive performance have not been well-defined (Morton, 2004).

Measurement of progesterone can be used as an aid to estimate the accuracy of estrus detection by determining whether the progesterone concentration at the time of insemination is high or low (Morton and Wynn, 2010;Ingenhoff et al., 2016). A high progesterone concentration indicates a cow being in diestrus or pregnant while a low progesterone concentration indicates a cow being in periestrus, estrus or indeed anestrus (Ingenhoff et al., 2016). As there is a strong correlation between milk and plasma concentrations of progesterone during the bovine estrous cycle (Dobson et al. 1975); radioimmunoassays (**RIA**) (Heap et al., 1976), enzyme-linked immunosorbent assay (**ELISA**) (Arnstadt and Cleere, 1981) and more recently, lateral flow enzyme immunoassays (Waldmann and Raud, 2016) have been developed for use on milk. Some of these more user-friendly assays have been marketed to farmers for on-farm use to assist in detection of non-pregnancy post-insemination or in the prevention of EDI at insemination (Waldmann and Raud, 2016).

The prevalence of EDI from different study populations varies markedly and Morton's review (2004) of the subject shows that it can range from 1-28% of all inseminations. This same review also noted a significant variation of the prevalence of EDI between herds [3.4-42.1%; (Reimers et al., 1985; Nebel et al., 1987] indicating that management factors may have a significant impact on EDI at a herd-level. Early studies from seasonal calving herds (Macmillan and Watson, 1976; Macmillan et al.,1977) and even more recent studies (Morton and Wynn, 2010), indicate that EDI is less of a problem in seasonal calving herds (< 5%) yet others have found much higher rates of EDI in these systems [11.1%; (Cavestany and Galina, 2001)]. Few studies have examined which cow-level risk factors may increase

the risk of EDI. Observed signs of estrus such as standing to be mounted (Reimers et al., 1985; Badinga et al., 1985), mounting others (Reimers et al., 1985) and mounting abrasions (White and Sheldon, 2001) have been associated with a reduced risk of EDI. Others have shown that the risk of EDI is increased if cows are inseminated when tail paint removal is the only observed sign of estrus (Ducker et al, 1983). Some authors have suggested that EDI can increase when farmers attempt to increase submission rates by submitting cows illustrating less predictive signs of estrus (Macmillan and Watson, 1976), while others have found no correlation, suggesting that improving one will not compromise the other (Morton and Wynn, 2010).

With expansion of dairy herd sizes over recent years in pasture-based systems (Dairy NZ, 2019; CSO 2019), the importance of achieving high submission rates (McDougall, 2006) and the seasonal demands of labor (O'Donovan et al., 2008), all mean that EDI in these systems could potentially be higher than previously studied. Consequently, the objectives were to 1) provide cow-level estimates of EDI prevalence and 2) determine cow-level risk factors for EDI.

5.3. Materials and methods

5.3.1 Relationship between foremilk and composite milk progesterone (validation study)

Progesterone is fat soluble and concentrations in milk are closely related to milk fat percentage (Pope et al., 1976). It is well established that milk fat percentage rises during milking (McCoy et al., 2001) and as farmers in our study relied on foremilk samples due to the parlor facilities (direct pipeline milking systems), we conducted a trial to standardize the relationship between progesterone concentrations, as estimated by our RIA, in foremilk and composite milk samples. This study was carried out at University College Dublin Lyons Farm using 160 samples collected every other day from 20 cows commencing on day of detected estrus until 14 days post estrus. Estrus was observed as cows standing to be mounted and confirmed by the presence of a dominant follicle(s) with no visible corpus luteum on either ovary using trans-rectal ultrasonography (Easi-Scan 1, 7.5-MHz linear-array transducer; BCF Technology, Mitcham, Australia). Briefly, foremilk samples were obtained before cluster attachment and after washing and drying the cow's teats, the first three draws of milk were discarded and thereafter at least 10ml of a foremilk sample (from two quarters or greater) was collected into sample pots containing preservative tablets (8mg bronopol/0.3 mg natamycin; Broad Spectrum Microtabs II, Advanced Instruments Inc., Norwood Massachusetts). The composite milk samples were collected from the milk meter toward the end of the same milking. After collection, milk samples were taken to the laboratory and frozen at -20°C until subsequent analysis with RIA (*see section 5.3.4 Sample testing*). The relationship between foremilk and composite milk progesterone was investigated with a linear mixed-effects model using the "lmer" function in "lme4" (Bates et al. 2015) in the R statistical programming language (R Core Team, 2015). As sampling was repeated in the same cow and taken over a sequential series of days from estrus to the mid-luteal phase and the relationship at any time point was of interest, both cow and day of sampling were treated as random effects. This relationship was investigated for linear and quadratic effects using "lmer" with the model that resulted in the lowest Akaike information criterion decided as the best fit. The result of the mixed model allowed prediction of a foremilk progesterone concentration equivalent to 5 ng/ml concentration in a composite milk sample as an indicator for active luteal tissue (Nebel et al.,1987; Nyman et al., 2018).

5.3.2 Study population

To identify the risk factors and prevalence of EDI, a cross-sectional study design was used. A sample of 20 herds within a one-hour drive of University College Dublin were selected (based in Co. Kildare, 52°60'N, 6°50'W; Co. Meath, 53°30'N, 6°32'W; and Co. Wicklow, 52°55'N, 6°25'W, Ireland). Of the 20 participating herds, 10 were exclusively spring calving (January to May) while the remaining 10 had distinct autumn (September to December) and spring calving (January to May) blocks. Cow breeds were predominantly Holstein-Friesian or Jersey crosses. Mean (standard deviation, **SD**) spring herd size was 192 (SD 70 range 82-350) cows, and mean parity was 2.8 (SD 1.7, range 1-12). Cows were housed in

cubicle sheds with slatted passageways during the winter (November to February), and were at pasture from mid-February until the end of October. During the winter, lactating cows typically received a mixed diet consisting of either grass silage (± maize silage) with concentrate supplementation in the parlor. Turn out to pasture for lactating cows typically occurred in mid to late February, when the main component of their diet was grass supplemented with concentrates in the parlor. Cows were milked twice per day. The per cow mean 305-d predicted milk yield, fat and protein was 6,689kgs (SD 767kgs), 304kgs (SD 60kgs) and 239kgs (SD 21kgs) respectively. As typical of seasonal pasture-based systems, all inseminations began on a farm specific fixed calendar mating start date (**MSD**) in either April or May and ceased on a fixed calendar date in July or August. Four of the participating herds used AI exclusively during the breeding season while the other 15 herds used AI initially followed by a period where stock bulls were introduced, and no further AI was conducted. The average length of the initial AI breeding period was 57.3 days (SD 17.4, range 32-87 days) while the subsequent stock bull breeding period lasted an average of 42.1 days (SD 13.8, 25-66 days) for those herds using bulls.

5.3.3 Sample collection

Prior to the MSD, all farms were visited and supplied with 60-90 sterile 20ml sample pots containing preservative tablets (8mg bronopol/0.3 mg natamycin). During this visit, farmers received formal instructions on how to correctly sample cows to standardize the sampling procedure. Farmers were instructed to sample cows on the day of AI by the same method as described in the validation study above. Sampling was conducted by one trained person per farm and continued until all the allocated sample pots were used, or until the period of artificial breeding ended. Prostaglandin induced estrus or hormone induced fixed-time AI were deemed ineligible for sampling. In addition, in herds with split calving blocks, only spring calved cows, were eligible for sampling. Samples were stored in the farm refrigerator and then collected on a weekly basis during the study period. The enrolled farms were requested to use their existing estrus detection aids and these included tail paint (n=12 farms), scratch cards (n=4 farms) and activity monitors (n=4 farms).

During the initial farm visit, farmers were instructed on how to record details relating to estrus and subsequent insemination on a standardized form. These included the type of estrus detection they used and the details of the observed estrus as follows; cow identification, date of service, inseminated after morning or evening milking, aid used to detect estrus, observation of standing to be mounted, observation of mounting behavior on other cows, presence or absence of external signs of mounting on the cow (abrasions of the tail head or tuber ischii) and the presence or absence of clear vaginal mucus. Milk sampling for this study was granted exemption from full ethical review by the Animal Research Ethics Committee (AREC-E-18-17-Beltman) in University College Dublin.

5.3.4 Progesterone analysis

Following collection, milk samples were taken back to the laboratory and frozen at -20°C until subsequent analysis. Milk samples were analyzed for progesterone concentrations using a validated RIA kit (DIAsource PROG-RIA_CT Kit; DIAsource ImmunoAssays S.A., Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium) previously described (Simersky et al., 2007) and adapted for milk. Briefly, defrosted milk samples, calibrators and controls were vortexed and 50µL of the milk samples, internal quality controls and standards were pipetted into antibody coated tubes. 500µL of Progesterone-I^{125} tracer was then added to every tube and the tubes were gently agitated and then transferred to a water bath at 37°C to incubate for 2 hours. The tube contents (except total counts) were aspirated, washed with solution, decanted, and dried. Lastly, the tubes were counted for one minute in a gamma counter. The intra-assay CV with internal quality control (**IQC**) samples for low, medium and high were 12.9%, 8.9% and 10.3% (10 assays, each with 10 replicates). The inter-assay CVs for the same IQC samples were 3.3%, 3.1% and 3.6%.

5.3.5 Fertility data collection

Calving and fertility data were recorded by farmers and entered the same on-farm software package (Irish Farm Computers, www.agrinet.ie). Information available on the sampled cows included the following: date of birth; parity; breed; preceding (2018) and subsequent (2019) calving date. Milk yields (305-d) were extrapolated using milk recording data available at the end of lactation (2018) from each cow. Predicted transmitting abilities (**PTAs**) for production and fertility were obtained from the Irish Cattle Breeding Federation (www.icbf.ie) and these were based on the assessment conducted on May 21st, 2019, for all cows. All inseminations (AI and bull-matings) recorded throughout the spring breeding season were obtained from the on-farm recording software. Routine pregnancy diagnosis for the cows was conducted by one of five veterinary operators using transrectal ultrasonography on routine fertility visits. The two visits took place in all eligible cows (inseminated ≥ 30 days) at 9-11 weeks after MSD and in all breeding cows 5-7 weeks after mating had ceased; including re-confirmation of pregnancy in those identified in-calf at the first visit (range of 35-150 days of gestation). The same operator conducted both visits used on each farm and they were experienced in ultrasonographic pregnancy diagnosis. The stage of gestation was estimated based on crown rump length or head diameter. Cows were censored from the descriptive statistics if their pregnancy status could not be determined due to the fact they were culled before sufficient pregnancy data were available.

5.3.6 Statistical analyses

All data manipulation and analyses were carried out using the R statistical programming language (R Core Team, 2015). An insemination and thus an estrus diagnosis, was defined as inaccurate if progesterone concentration in foremilk samples was greater than or equal to 3 ng/ml based on the

validation study results. Cow-level prevalence of EDI was thus reported as the proportion of insemination milk samples ≥ 3 ng/ml over the total milk samples gathered.

The response variable used for the cow-level risk factors was binomial, with inseminations being classified as accurate (low progesterone; < 3 ng/ml) or inaccurate (high progesterone; ≥ 3 ng/ml). As initial univariate analysis showed that repeat inseminations were at a much higher risk of being inaccurate (OR = 4.6; P <0.01), the fact some of the study farms (n=6) only sampled first inseminations and that the risk of inseminating a pregnant cow is impossible on a first insemination, we decided to stratify analysis by insemination number (first or repeat) to further investigate the risk factors and thus built two separate models. The categorical risk factors investigated were as follows: parity (1 to ≥4); breed (coded as Holstein Friesian, (>11/16) or crossbred (>85% of which were Jersey cross)); standing to be mounted (observed or not observed); mounting of other cows (observed or not observed); mounting abrasions of the tail head or tuber ischii (observed or not observed); clear vaginal mucus (observed or not observed); time of day of insemination (morning or evening); and only for the repeat model; abnormal preceding repeat interval (abnormal; (3-17 days and 25-35 days) vs normal (18-24 days)). Continuous risk factors investigated were as follows: DIM at insemination; 305-day energy corrected milk yield in kilograms based on the formula by Sjaunja et al.(2000); and genetic PTAs for production (kilograms of milk, fat and protein) and fertility (calving interval and survival). All continuous covariates were standardized by subtracting the observed value from the mean and then dividing by the standard deviation. All standardized continuous covariates were then tested as both linear and quadratic covariates. The individual explanatory covariates were initially investigated in a univariate logistic regression framework stratified by insemination number with herd as a random effect ["glmer" function in "lme4" (Bates et al., 2015)] and then carried forward for multivariate regression analysis when P < 0.2. All candidate covariates for multivariate modelling were assessed for correlation. When two variables were highly correlated (>0.8), one was selected and brought forward for multivariate analysis based on the variable that resulted in the model with the lowest Akaike information criterion. Variables dropped due to collinearity were replaced in the final model to test for significance. A model was constructed using forward stepwise elimination, and variables with a significance probability of P < 0.05 retained in the final model. Herd was treated as a random effect as sampling was clustered within herd and cow-level risk factors for EDI were the variables under investigation. All 2-way interactions were assessed and retained in the model if P < 0.05. The models produced adjusted odds ratios (AOR), which served as an approximation of the association between the risk factors and EDI.

5.4 Results

5.4.1 Relationship between foremilk and composite milk progesterone

In the validation study, the relationship between foremilk and composite milk progesterone estimated by the mixed linear regression model is shown below where; y = foremilk progesterone (ng/ml), 1.27 = the intercept, 0.35 =slope or Beta, x = composite milk progesterone concentrations (ng/ml):

$y = 1.27 + 0.35x$

The r^2 value is the percentage of the response variable variation that was explained by a linear model and in the case of this trial, this was approximately 85%. Based on the linear mixed-effects model, a foremilk progesterone concentration of 3 ng/ml corresponded to a milk progesterone concentration of 5 ng/ml in composite milk using this RIA.

5.4.2 Descriptive statistics

A total of 1071 milk samples were collected from 984 cows on 19 farms between the 9th of April and 19th of June 2018; during this period, only artificial insemination was used on the farms. Samples collected from an additional farm were excluded entirely from RIA analysis as too few cows were sampled, and record keeping was generally poor. Other samples that were excluded, included 7 samples that had no recorded insemination relating to the sample and 1 sample that was not of suitable quality for analysis. 56 duplicate milk samples were taken at double inseminations, either as a morning-evening or an evening-morning insemination. As these samples were taken from the same estrous period the milk sample with the lowest progesterone was retained for further analysis (n=28 samples). Thus, 1035 milk samples from 977 cows at insemination in 19 herds were retained for further analysis.

The average DIM at sampling was 75.2 DIM (SD 21.6; range 15-135). The average sampling period varied with a mean of 27.4 days (SD 14.1, range 5-50) and during this period a mean of 35.5% (SD 15.8%, range 14.7-77.6%) of all artificial inseminations were milk sampled.

Of the signs of estrus recorded by the farmer, mounting abrasions were the most prevalent sign (83.2%; 861/1035) recorded, followed by the observation of standing to be mounted (41.3%; 427/1035), mounting other cows (27.8%; 287/1035) and clear vaginal mucus discharge (18.2% ; 188/1035).

The total number of milk samples with high progesterone (≥ 3 ng/ml) was 49 samples (4.7%) and with low progesterone was 986 (95.2%). Of 1035 milk samples, 900 samples from 19 farms were from first inseminations and of these, 30 samples (3.3%) had high progesterone. 135 samples of repeat inseminations were collected on 13 farms and of these, 19 samples (14.1%) had high progesterone. In total, 57 cows had milk samples collected at both their first and repeat inseminations. A frequency distribution of the foremilk progesterone concentrations of the samples collected at the time of insemination is presented in Figure 5.1.

Of the 977 cows milk sampled, pregnancy status could not be determined for 52 cows because either the cow was not pregnancy tested or a pregnancy testing result was not recorded. Retrospective analysis of the breeding records revealed that the pregnancies per AI to samples with low progesterone (mean concentration 1.07ng/ml) was 54% (507/931) while the pregnancies per AI to samples with high progesterone (mean concentration 9.20 ng/ml) was 2.5% (1/49). 9 of the 19 (47%) cows found to have high progesterone levels at the time of repeat insemination were subsequently found to have conceived to a previous insemination.

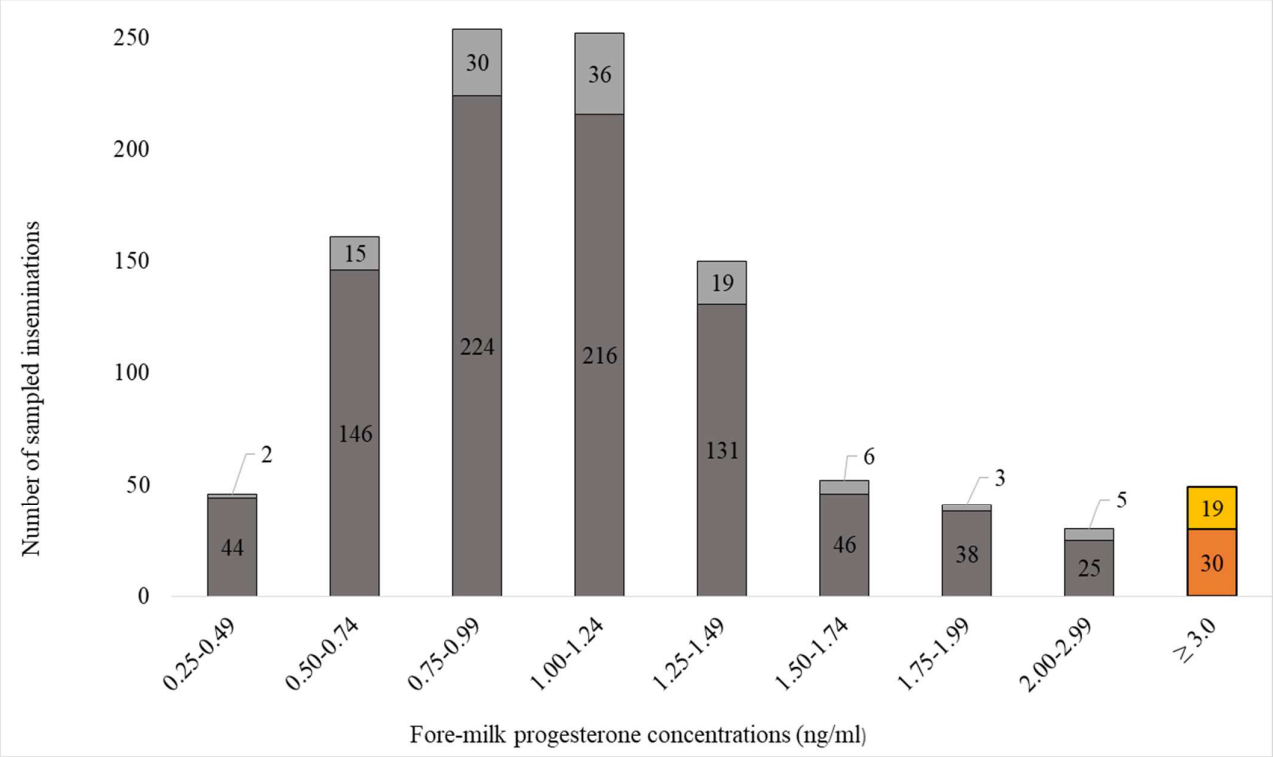

Figure 5.1 Frequency distribution of the foremilk progesterone concentrations of the samples collected at the time of insemination. The dark grey bars (n=870) are first insemination that were classified as accurate (<3 ng/ml) while the light grey bars (n=116) are repeat inseminations that were classified as accurate. The orange (n=30) and yellow (n=19) represent the samples that were classified as inaccurate (≥ 3 ng/ml) for first and repeat inseminations respectively. (Supplemental to published research).

5.4.3 Risk factor model outcome

Data were stratified by insemination number (first or repeat) to further investigate the risk factors for both groups, and thus two separate models were built. The outputs from the univariate and final multivariate logistic regression models for first and repeat inseminations are presented in Tables 5.1 and 5.2, respectively. For first inseminations, the odds of the EDI were increased if mounting abrasions were absent on a cow (AOR; 3.0; $P < 0.01$). For repeat inseminations, the odds of EDI were increased if there was the absence of mounting abrasions (AOR = 4.1; $P < 0.05$), the interval preceding the repeat insemination was abnormal (AOR; 9.5; $P < 0.01$) or if no standing estrus was observed (AOR; 12.5; $P < 0.05$).

Table 5.1. Univariate and multivariate results for risk factor estimates for inaccurate estrus on first insemination from 19 farms with herd included as a random effect (n=900).

			Individual Risk Factors			Final Model		
Categorical variables	**Class**	**n[1]**	**AOR[2]**	**95% CI**	**P-value**	**AOR[2]**	**95% CI**	**P-value**
Parity	1	270	Referent			-	-	-
	2	211	1.2	0.5-3.5	0.669	-	-	-
	3	144	0.7	0.2-2.8	0.626	-	-	-
	4+	275	1.4	0.5-3.6	0.491	-	-	-
Breed	JEX	205	Referent			-	-	-
	HF	695	1.2	0.5-3.3	0.663	-	-	-
Standing Estrus Observed	Yes	368	Referent			-	-	-
	No	532	2.0	0.8-4.9	0.147	-	-	-
Mounting of herdmates Observed	Yes	260	Referent			-	-	-
	No	640	0.7	0.3-1.5	0.344	-	-	-
Tail or Rump Abrasions	Yes	757	Referent			Referent		
	No	143	3.0	1.3-6.9	<0.01	3.0	1.3-6.9	<0.01
Clear Mucous Discharge	Yes	157	Referent			-	-	-
	No	743	3.8	0.8-19.0	0.101	-	-	-
Time of AI	AM	683	Referent			-	-	-
	PM	217	3.1	0.6-3.1	0.452	-	-	-
Continuous Variables		**n[1]**	**AOR[2]**	**95% CI**	**P-value**	**AOR[2]**	**95% CI**	**P-value**
DIM at sampling	-	-	0.8	0.6-1.2	0.297	-	-	-
305-day ECM yield	-	-	0.6	0.4-1.1	0.103	-	-	-
Milk PTA	-	-	0.9	0.6-1.3	0.554	-	-	-
Fat PTA	-	-	0.9	0.6-1.2	0.421	-	-	-
Protein PTA	-	-	0.9	0.6-1.4	0.735	-	-	-
Calving Interval PTA	-	-	1.3	0.9-1.9	0.202	-	-	-
Survival PTA	-	-	0.9	0.6-1.3	0.496	-	-	-

Table 5.1 Footnotes

[1] n = number in category.

[2] AOR = Adjusted Odds Ratio

Table 5.2. Univariate and multivariate results for risk factor estimates for inaccurate estrus on repeat inseminations from 13 farms with herd included as a random effect (n=135).

			Individual Risk Factors			Final Model		
Categorical variables	**Class**	**n[1]**	**AOR[2]**	**95% CI**	**P-value**	**AOR[2]**	**95% CI**	**P-value**
Parity	1	38	Referent			-	-	-
	2	35	1.8	0.5-6.6	0.383	-	-	-
	3	14	1.2	0.2-7.2	0.859	-	-	-
	4+	48	0.8	0.2-2.6	0.762	-	-	-
Breed	JEX	27	Referent			-	-	-
	HF	108	0.7	0.2-3.0	0.862	-	-	-
Standing Estrus Observed	Yes	59	Referent			Referent		
	No	76	8.2	1.8-37.2	<0.01	12.5	1.6-99.8	0.017
Mounting of herdmates Observed	Yes	27	Referent			-	-	-
	No	108	1.0	0.3-3.4	0.994	-	-	-
Tail or Rump Abrasions	Yes	104	Referent			Referent		
	No	31	3.8	1.4-10.7	0.011	4.1	1.1-14.6	0.031
Clear Mucous Discharge	Yes	31	Referent			-	-	-
	No	104	0.6	0.2-2.3	0.459	-	-	-
Time of AI	AM	118	Referent			-	-	-
	PM	17	3.1	0.7-13.9	0.141	-	-	-
Abnormal Preceding Service Interval	No	99	Referent			Referent		
	Yes	37	7.3	2.3-23.0	<0.01	9.5	2.4-37.1	<0.01
Continuous Variables		**n[1]**	**AOR[2]**	**95% CI**	**P-value**	**AOR[2]**	**95% CI**	**P-value**
DIM at sampling	-	-	1.0	0.6-1.6	0.941	-	-	-
305-day ECM yield	-	-	0.5	0.2-1.2	0.117	-	-	-
Milk PTA	-	-	1.0	0.6-1.6	0.857	-	-	-
Fat PTA	-	-	0.7	0.4-1.2	0.162	-	-	-
Protein PTA	-	-	0.9	0.5-1.4	0.611	-	-	-
Calving Interval PTA	-	-	1.1	0.7-1.9	0.685	-	-	-
Survival PTA	-	-	1.5	0.9-2.5	0.160	-	-	-

Table 5.2 Footnotes

[1]n = number in category.

[2]AOR = Adjusted Odds Ratio

5.5 Discussion

The results of the validation study determined that a strong correlation existed between progesterone concentrations in composite and foremilk (r=0.92). The lower progesterone concentrations in the foremilk samples compared with their corresponding composite milk samples was likely due to the lower fat in foremilk samples compared with composite samples, a finding noted by others (Heap et al., 1976, Pope et al., 1976) . The r^2 value between foremilk and composite milk progesterone concentrations was similar between this validation study (0.85) and another study (0.83) that used an ELISA progesterone assay (McCoy et al., 2001).

The relatively low prevalence of EDI (4.7%) calculated in the present study indicates that estrus detection by farmers in this sample of Irish seasonal calving herds was good. It differed from previous reported studies that have shown EDI prevalence of above 10% (Appleyard and Cook 1976; Sturman et al., 2000) but was in agreement with other more recent studies (3.0% (Ingenhoff et al., 2016)). The EDI prevalence in the present study was also in agreement with the majority of studies in seasonal breeding systems [3.4% (Morton and Wynn, 2010)] and had a lower prevalence than previous studies in Ireland [19.7% (White and Sheldon, 2001)]. Morton's review (2004) had noted a difference in the estimated EDI prevalence between seasonal calving herds and year round systems but subseqently found that EDI prevalence was generally low in both systems during his own investigations (Morton and Wynn, 2010). It is possible that if a difference does exist, it may be explained by inherent factors in theses types of herds such as herd mangement (e.g., housed vs at pasture) during the breeding season or the larger sexually active group seen in seasonal breeding systems. However, in a study investigating the methods of estrus detection between housed and pasture-based spring calved cows, Palmer et al. (2010), showed that although all estrus detection methods tested had a higher efficiency in cows at pasture than in housed cows, there was no difference in the accuracy of estrus detection between the two management groups.

The prevalence of EDI in the present study differed ($P < 0.01$) between first (3.3%) and repeat inseminations (14.1%) based on initial univariate analysis. This contrasts with what has been noted in other studies which have described that the EDI prevalence does not differ between first and repeat insemination (Rajamahendran et al., 1993) or others that indirectly claim in seasonal herds there is a greater tendency for farmers to have increased EDI at first as opposed to second inseminations (Macmillan et al., 1976). This could be due to a higher rate of insemination rejection by AI technicians or farmers in these studies. Sturman et al. (2000) has noted that up to 16% of re-inseminations are rejected by AI techicians in Israel, something that may not be happening in our study population.

There was a difference ($P < 0.05$) in EDI prevalence between farms in our initial univariate investigations and this has been noted by others (Reimers et al., 1985; Nebel et al., 1987). The overall within herd prevalence of EDI ranged from between 0-15.6% illustrating that errors in estrus detection can be a herd specific problem. Morton and Wynn (2010) noted that although the EDI prevalence was low (3.4%) in seasonal calving herds, 25% of these herds had a prevalence of greater than 5% which was similar to

the 26% of herds in our study indicating that it could be an issue in a proportion of spring calving herds. However, at this point, it is worth stating that establishing herd-level estimated prevalence or risk factors were not aims of this study and herd was treated as a random effect in the risk factor modelling. Further research to identify herds with EDI problems and the associated risk factors within these herds is likely to be beneficial to assist these farmers.

Mounting abrasions of the tail head or tuber ischii were the most prevalent sign of estrus (83.2%) observed by the farmers in the present study. The prevalence of cows observed in standing estrus was lower (41.3%) than what has been previously observed in pasture based dairy cows [59% (Palmer et al., 2010)] but was comparable with other studies [37% (Van Eerdenburg et al., 1996) and 43% (LeRoy et al., 2018)]. Cows on pasture or earth have more standing estrus events than cows housed on concrete (Palmer et al., 2010), but the proportion observed is highly dependent on the frequency and duration of observation (Palmer et al., 2010). As this varied considerably between the farms in the present study, it may explain the lower-than-expected prevalence of observed standing estrus events. Clear vaginal discharge was the least prevalent observed sign of estrus and this was comparable with other studies that have noted that it was not consistently observed around the time of estrus (Van Eerdenburg et al., 1996; LeRoy et al., 2018).

The only risk factor for EDI at first insemination identified in the present study was the absence of mounting abrasions (AOR; 3.0; $P < 0.01$) and this has been noted by others (White and Sheldon, 2001). Interestingly, in contrast to this, others have shown that the risk of EDI was increased if cows were inseminated where tail paint removal was the only observed sign (Ducker wt al., 1983). However, on the present study farms and in other studies, reasonable pregnancies per AI were achieved in cows that were inseminated without being seen standing to be mounted but with other signs of estrus (Reimers et al., 1985; Badinga et al., 1985). Additionally, tail paint removal and hip abrasions are not necessarily synonymous thus our finding is consistent with previous research. Based on the present study findings, the absence of mounting abrasions may facilitate the strategic use of on-farm progesterone assays at first inseminatons. However, what was also noteworthy from the present study, was that the majority (73%; 22/30) of cows that had high progesterone on first insemination repeated in a short inter-insemination interval of 4-17 days. Many authors have described that short repeat ('interestrual') intervals <18 days exist as a result of EDI (Macmillan and Watson, 1976; Reimers et al., 1985) but other causes of short interestrual intervals also exist and include; cystic ovarian disease or genuine short cycles (Macmillan and Watson, 1971). Other authors (Macmillan et al., 1977) have reported that insemination of a cow inaccurately diagnosed in estrus has no effect on the pregnancy per AI to a subsequent accurate insemination. Although it was not possible in the present study to determine if all of these subsequent short repeat intervals were accurate, the effect that this inaccurate first insemination would have on days open in the breeding season was likely to be negligible thus putting into question the cost-benefit of even strategic milk progesterone testing based on the absence of mounting abrasions.

Absence of mounting abrasions (AOR; 3.0; P < 0.01) were also a risk factor for EDI at repeat inseminations but additional risk factors were also identified. For repeat inseminations, cows with an abnormal preceding interval (AOR; 9.5; P <0.01) prior to the repeat insemination were at increased risk of EDI. Examination of inter-insemination interval is a well-established method at both cow-level and herd-level to detect potential inaccurate estrus detection on farms (Sturman et al., 2000) and the results of our study supports this practice. In the case of repeat inseminations, analysis of breeding records should be conducted to detect any abnormal preceding interestrual intervals (either of 3-17 or 25-35 days) to target these cows for progesterone testing. Abnormal preceding interval was not assessed on the model of first insemination in our analysis as no pre-breeding estrus detection records were available for the enrolled farms, so it is possible that it may have been a risk factor for first inseminations had farmers been recording these. As in other studies (Reimers et al., 1985; Badinga et al., 1985), cows that had no observed standing estrus (AOR; 12.5; P <0.05) were also at increased risk of EDI at a repeat insemination and thus could be targeted for on-farm milk progesterone testing. Many studies have illustrated that both duration and intensity of estrus increases as the numbers of cows in the sexually active group increases (Roelofs et al., 2005). In seasonal pasture-based systems, this sexually active group is largest in the first three weeks, but this reduces in each subsequent three-week block as cows become pregnant. Therefore, as estrus events decline and the number of cows that are pregnant increases within the herd after the first 3 weeks, it was only logical to assume that the odds of EDI increase. Our finding of an increased risk of EDI in cows that showed no observed standing estrus suggests that increasing visual estrus detection frequency and duration during the second three-week block of the breeding season may aid in its reduction.

In the present study, 47% of cows with high progesterone on repeat insemination were found to have conceived to a previous insemination. Ingenhoff et al. (2016) found that 50% of cows with high progesterone at the time of insemination were pregnant while Sturman et al. (2000) has documented that 45.5% of cows that were rejected by AI technicians in Israel were subsequently pregnant both of which are similar to our findings. Insemination of pregnant cows has the potential to result in the loss of the pregnancy. Estimates of the percentage of pregnancy losses due to an inaccurate insemination vary from 13-29% (Macmillan et al., 1977; Sturman et al., 2000). This largely depends on the number of days post-insemination when an inaccurate insemination occurs (Macmillan et al., 1977) and the site of semen deposition in the reproductive tract; intrauterine versus mid-cervical (Weaver et al., 1989). 44% of the pregnant cows on this study were inseminated at greater than 17 days since the previous insemination and some authors have suggested when insemination occurs after these days that there may be a lower probability of pregnancy loss (Macmillan et al., 1977). Mid-cervical insemination on repeat insemination has been shown to have a lower probability to cause pregnancy loss versus intrauterine insemination (Weaver et al., 1989) but site of insemination was not determined in the present study. In general, due the nature of how routine pregnancy diagnosis is conducted in seasonal systems, estimating the pregnancy loss is difficult and was beyond the scope of this study. This is because cows are evaluated

at different time points after artificial insemination or bull mating, and bull matings are not always recorded. Although it was not possible to assess pregnancy loss due to inaccurate repeat inseminations in the present study, 45% of repeat inseminations that had high progesterone were found to be subsequently pregnant. This suggests that even with conservative estimates of pregnancy loss, the proportion of cows that had high progesterone at repeat insemination and were pregnant were likely underestimated in this present study.

Studies where EDI is estimated based on milk sampling for progesterone concentrations on the day of artificial insemination have certain limitations. One of these is that the prevalence of EDI may be underestimated in cases where cows in either anestrus or periestrus are inseminated. In both cases, sampling at the time of insemination will yield a low milk progesterone value which will be erroneously recorded as a true estrous event. In a review of the postpartum anestrous interval (Rhodes et al., 2003), it was estimated in seasonal calving dairy herds that anywhere between 13 and 48% of cows are diagnosed as being anovulatory anestrus in the week prior to the breeding period while others, have found that the mean calving to first ovulation in pasture based cattle was 43.4 days (McDougall et al., 1995). In the present study, the mean DIM at sampling was 75.2 with less than 10% of samples taken before 42 DIM, therefore it unlikely that many anestrous cows were present in our sampled cohort. Similarly, insemination of cows during periestrus will also lead to underestimation of EDI prevalence, these cows will have a low milk progesterone despite sub-optimal timing of insemination. The proportion of cows inseminated during periestrus and erroneously categorised as an appropriate insemination could vary depending on the progesterone cut-off used. Interestingly, even with the progesterone threshold concentration used in the present study, one cow sampled with high progesterone at the time of insemination did conceive to the sampled serve. Cows conceiving with elevated milk progesterone have been documented previously (Sturman et al., 2000; Ingenhoff et al., 2016) and can be explained by sampling or assay error or even extra-ovarian sources of progesterone such as the adrenal glands (Bolanos et al., 1997).

On-farm progesterone assays of cows undergoing artificial insemination, or in-line milk progesterone measurement of cows are not commonplace in pasture-based herds. This likely owes to the fact that both can be costly methods for heat detection either through investment and maintenance in the case of fully automated milking systems or due to low degrees of automation and high costs associated with the use of individual cow progesterone testing (Yu and Meada, 2017). Ingenhoff et al. (2016) found that routine testing using a milk progesterone ELISA for all cows diagnosed in estrus is not economically justified to detect EDI but instead suggested a more strategic use of on-farm milk progesterone testing. Although this present study did not simulate an on-farm cow situation, the findings are relevant and likely to assist in further studies into the economic and practical feasibility of strategic use of on-farm milk progesterone testing based on simple and practical cow level risk factors to detect EDI.

5.6 Acknowledgements

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Chapter 6

General discussion, conclusions and future research

6.1 General conclusions and discussion

As detailed discussion of each individual study is presented within their respective chapter, the objective of this chapter is to present a concise summary of the main conclusions arising from the research presented in this thesis. It will aim to explain how the results of these studies in combination with other information in the literature may inform practical recommendations to Irish farmers, veterinarians and other advisors who deal with reproduction management of seasonal calving pasture based dairy cows. Lastly, this chapter will aim to outline areas of further research that would be useful relating to the main areas of research. These areas will be discussed separately in relation to the two research themes.

6.2 Main conclusion drawn from studies relating to the reproductive tract disease research

The main conclusion on reproductive tract disease from Chapter 2 of the thesis were:

- Both methods of diagnosis (i.e. PVD and UE scoring) shared risk factors and these included;
    - The days in milk when the pre-breeding examination was performed
    - The retention of fetal membranes after calving
    - The occurrence of a twin birth at the last calving
    - The genetic value for fertility of a particular cow.
- However, it also illustrated that other risk factors appeared specific to diagnostic methods assessed;
    - For positive PVD diagnosis those were;
        - Dystocia at the last calving
        - Breed of the cow (i.e., HF cows)
        - Primiparity (i.e., 1st lactation cows)
        - Poor (<2.75) BCS at the time of the pre-breeding examination
    - For positive UE scoring those were;
        - Older parity (≥5 parity)
        - Beef sired calf born at the previous calving
        - The absence of a corpus luteum at the time of the pre-breeding examination.
- The shared and varied risk factors between both diagnostic methods may indicate that in some cases the diagnostic methods are diagnosing separate components of reproductive tract disease, such as cranial vaginitis, cervicitis, clinical or subclinical endometritis as well as pyometra.
- However, that said, all risk factors identified should be considered important in their ability to increase the probability of reproductive tract disease.

The main conclusion on reproductive tract disease from Chapter 3 of the thesis were:

- Dairy cows that were diagnosed with reproductive tract disease by with either visual assessment of vaginal discharge (i.e., PVD scoring) or ultrasonographic assessment of intrauterine fluid (i.e., UE scoring) were associated with impaired reproductive performance.
    - For PVD scoring those cows that had discharge that was mucopurulent or worse (i.e., score of ≥ 2 on the 0 to 3 scale) were associated with impaired reproductive performance.
    - For UE scoring those cows that had greater than or equal to trace intraluminal fluid present (i.e., score of ≥ 1 on the 0 to 5 scale) were associated with impaired reproductive performance.
    - The time of diagnosis relative to when breeding commences may also influence the threshold deem appropriate to consider positive and treat with cows that have flecks of purulent material that is detected with less than 3 weeks to MSD potentially having an argument for receiving a treatment due to impaired reproductive performance.
- The optimal threshold for diagnosing reproductive tract disease with either diagnostic test at the time of sampling (i.e., the pre-breeding examination) were;
    - Cows that had discharge with flecks of purulent material or worse visible (i.e., score of ≥ 1 on the 0 to 3 scale) for PVD scoring.
    - Cows that had greater than or equal to trace (≥2 mm) intraluminal fluid present (i.e., score of ≥ 1 on the 0 to 5 scale) for UE scoring.
- While both diagnostic methods were associated with impaired reproductive performance transrectal ultrasonographic assessment of intrauterine fluid (i.e., UE scoring) appeared to have better test sensitivity and specificity estimates for the complete dataset. However, these test parameters were affected by days in milk when the pre-breeding examination occurred and should be considered when selecting a diagnostic test.

6.3 Practical implications of findings to Irish farmers and advisors relating to the reproductive tract disease research in this thesis

The identification of cow and calving related risk factors, determining the thresholds that are associated with reduced reproductive performance and better understanding test sensitivity and specificity parameters in relation to reproductive tract disease have practical implication for farmers and their advisors. These implications relate to following areas and are discussed below individually in relation to the conclusions;

- Identifying at risk cows and implementing preventive measures
- Decisions relating to which cows to examine?
- Decisions relating when to perform examinations?
- Decisions relating which diagnostic tests to choose?
- Decisions relating to which cows to treat?

6.3.1 Identifying at risk cows and implementing preventive measures

Farmers and advisors could use some of the information generated from the risk factor study in combination with existing knowledge and advice from the research literature to identify at risk cows and implement simple preventive measures to reduce of reproductive tract disease at cow-level. From a farmer's point of view, the risk factors that are most useful to know about are those that are easily modifiable. However, as is the case with many of the of the risk factors identified in both in this thesis and the literature few, of them are easily modifiable at cow-level ,e.g., a farmer can do little to change an individual cow's parity, her breed, her calving date, whether or not she becomes pregnant with twins or her genetic make-up. That said, knowledge of some of the same risk factors may be of use as management changes in certain cohorts of cows should help reduce the likelihood of reproductive tract disease development. Minimizing metabolic and calving related issues in these cohorts is likely to make the greatest benefit in reducing reproductive tract disease. Some of the more practical recommendations to farmers originating from the study results and a review of other literature around preventative measures are made below.

- Attempts to reduce the probability of retained fetal membranes or mitigate its effect on reproductive tract disease development might include:
    - Ensuring nutritional management (i.e., appropriate diet energy density, macro- and micronutrients supplied, feed space requirements as well as water supply) over the dry period is optimised (Gilbert, 2016).
    - Minimising dystocia by careful breeding decisions (see later).
    - Keeping adequate records of cows that have retained fetal membranes so pre-breeding examinations can be planned.

    - Monitoring for signs of metritis and generalized illness in cow with retained fetal membranes and treating promptly and appropriately (Gilbert, 2016).
- Attempts to mitigate the effect of twin births on reproductive tract disease development might include:
    - Identifying cows pregnant with twins at routine pregnancy diagnosis to allow for advanced planning for calving (Fricke, 2001). Be aware this can be a technically more difficult issue when pregnancy diagnosis is performed in later gestation as is common in many seasonal herds.
    - Being aware of the likely shorter gestation to plan earlier dry-off and skilled calving supervision (Bell and Roberts, 2007).
    - Managing those carrying twins separately from a nutritional point of view over dry period to minimize BCS loss (Bell and Roberts, 2007).
    - Potential administration of a monensin bolus 3-4 weeks prior to calving to minimize metabolic issues (Atkinson et al, 2015). Although, licensed for the purpose of ketosis prevention in Ireland, the use of monensin is controversial as it may be deemed a prophylactic use of an antimicrobial.
    - Attempts to reduce double ovulation and twinning are challenging practically on farms and methods of pregnancy termination or selective reduction (Cabrera and Fricke, 2021) are likely not useful in seasonal systems as the chance to re-conceive following a loss of pregnancy is restricted by the breeding season. Therefore these are not recommended.
- Attempts to reduce the probability of dystocia or mitigate its effect on reproductive tract disease development might include:
    - Careful and skilled assistance at calving time so a dystocia can be dealt with promptly. This is particularly important in primiparous cows (Mee, 2004).
    - Careful sire selection prior to breeding to minimize dystocia at the subsequent calving. This is especially relevant in replacement heifers.to minimize risk of dystocia at calving (Mee, 2004).
    - Ensuring replacement heifers are of adequate bodyweight at mating (Mee, 2004).
    - When using beef sires attempt to select for breed with a lower dystocia value (Mee, 2004).
- Attempts to reduce the BCS loss or extremes of BCS or mitigate its effect on reproductive tract disease development might include:
    - Regular monitoring of BCS to identify cows that are under or over targets. Both late lactational and dry cow BCS monitoring is critical to make changes to ensure cows calve at adequate BCS (3.0-3.25) (Buckley et al. 2003; Roche et al., 2013b).

- Ensuring nutritional management (i.e., diet energy density, macro- and micronutrients and feed space) in their dry, transitional and early lactational diet is optimized (Roche et al., 2013b).
- For individual at risk cows (i.e., fat or thin cows at calving) careful monitoring to ensure that there is not excessive or further loss of BCS loss and consider strategies such as extra supplementation or once- a-day milking (McNamara et al., 2008) until breeding if feasible.
- Promptly identify ill cows at calving or in early lactation (e.g., cows with displaced abomasum, ketosis etc.) and treat promptly and appropriately.

- Attempts to improve genetics for fertility may mitigate its effect on reproductive tract disease development.
  - Consider sires with a higher fertility subindex in cows that have lower than average values, this will ensure their replacement offspring will be at lower risk of reproductive tract disease and of better overall reproductive performance.
- Little will alter parity or calving date and thus considering culling those cows at 5th parity or above and those cows calving late in the season is worth strong consideration prior to breeding.
- If breeding in later calving cows is a must, implementation of synchronization regimes may be of benefit as it should advance calving date the subsequent year (Herlihy et al., 2011).

6.3.2 Decisions relating to which cows to examine

As the diagnosis of reproductive tract disease involves examination by a trained professional, it incurs an expense to the farmer, especially if the farmer opts to examine every cow that they intend to breed. However, the use of farm records may decrease the cost of diagnosis and increase the cost: benefit to the farmer by targeting cows with some of the risk factors identified in Chapter 2. It is also important to remember that by applying this approach of using historical records to identify cows is essentially similar to a 'screening' test and then the diagnostic test applied to them is the 'confirmatory' test (McDougall et al., 2007). This testing strategy therefore is similar to the testing in series approach, i.e., the approach of re-testing cow with positive test results (i.e., assuming the condition is present based on the risk factors) versus test in-parallel approach (see section 6.4). This approach will result in loss of diagnostic sensitivity compared to a single test but a net gain in specificity (Dohoo et al., 2003). In other words, it would reduce the risk that cows without reproductive tract disease would be diagnosed incorrectly and treated, but also it would increase the risk that a truly infected cow would be missed. As already stated if a farmer fails to keep accurate historical records especially relating to calving and the other risk factors identified in this thesis then the 'sensitivity' of this screening test could be low, so this is important to stress. That stated some of the practical recommendations regarding cows to select for examination based on this research might include;

- Cows that have retained fetal membranes and/or given birth to twins should be recorded and then marked for examination prior to breeding.
- Currently, the number of births and calving difficulty is mandatory (i.e., for calf registration) for recording on all Irish farms but recording retained fetal membranes is not. Considering the importance of this risk factor, it is suggested that this becomes an additional mandatory part of calf registration. This would be useful for farmers at a cow-level and for monitoring at a national level also.
- Cows that are calving cows closer to the MSD (e.g., cows that are likely between <40-35 DIM by MSD). This might also assist in decision relating to whether they should be bred or not.
- Although some of the risk factors were identified for specific diagnostic tests used, considering other literature reviewed (section 1.2.8), it would also seem reasonable to select the following cows for examination;
    - Those that experience a calving difficulty score of 3 or greater [i.e., recorded as considerable difficulty or veterinary assistance on the mandatory ICBF monitoring scoring system scale of 1-4 (Mee et al., 2011)].
    - Those cows in poor BCS (<2.75) or losing excessive BCS (>0.5) if routine BCS records are available.
    - Those cows that have not been observed in estrus if pre-breeding estrus detection is conducted on the farm.

6.3.3 Decisions relating to when to perform examinations

The next question that arises is when the examinations should be performed. Typically, pre-breeding examination of all cows are performed within the month prior to breeding and in the dataset analyzed in this study the median (interquartile range) DIM was 49 (21) days which is quite typical and similar to other work published in Ireland (Mee et al., 2009). From Chapter 3, that DIM had an influence on the test sensitivity, i.e., it was decreased for both tests as the DIM increased. Likewise in Chapter 2, there was an interaction between DIM at examinations and retained fetal membranes and the presence of the corpus luteum at the pre-breeding examination which altered the predicted probability of detection with the diagnostic tests. In this case based on these results, it might seem reasonable that earlier examinations should take place, particularly in at-risk cows identified. Based on these finding the practical recommendations regarding when to perform the pre-breeding examination might include;

- Pre-breeding examinations might be more beneficial if there were conducted over multiple visits prior to breeding and aimed at a reduced subset of at-risk cows opposed to a single visit of multiple cows close to MSD. Multiple visits would be more expensive but should be offset by the reduced numbers of cows examined per visit.

- An earlier visit/visits during latter part of the calving season (e.g., early-mid March) which would focus on cows that calved early (and are ≥25 DIM) but had issues such as retained fetal membranes, twins, dystocia and were in poor BCS. This would have the added advantage of re-examining cows with abnormalities detected at a later visit.
- A later visit/visits toward the end of the calving season and closer to MSD of cows not examined at the first visit(s) that had all the same issues as listed above but are only now eligible for examination (i.e., now ≥25 DIM). This examination would also include cows not observed in estrus if pre-breeding estrus detection is practiced, and those cows that noted for re-examination at a previous visit.

6.3.3 Decisions relating to which diagnostic tests to choose

The last question that arises is which of the diagnostic tests assessed should be used to screen for reproductive tract disease. In order to answer this for farmers, there are practical factors outside of the research results that are worth first considering. First of all, both tests described in this thesis have the advantage of being rapid cow-side tests with immediate results, having minimal risk to the cows in terms of contamination of the reproductive tract and requires no further processing unlike subclinical endometritis assessments (section 1.2.9). However, there are some important differences also between these tests to consider. Firstly, using of the Metricheck® device alone can rapidly screen a large number of cows for disease and can be performed by any personnel once appropriately trained so its cost of diagnosis per cow is likely to less than transrectal ultrasonography or other diagnostics. Transrectal ultrasound is not as rapid and it requires a more appropriately trained professional (most often veterinarians) and thus it is more expensive per cow. However, it has the distinct advantage of detecting any other abnormalities of the reproductive tract such as cystic ovarian disease that vaginal discharge assessment with a Metricheck® alone will not detect. Based on the data presented in this research it appeared that transrectal ultrasound alone had better test sensitivity when compared to vaginal discharge assessment with a Metricheck® over the complete dataset analyzed. However, it must be it must be emphasized that this higher test sensitivity was dependent on the DIM when the pre-breeding examination is taking place with test sensitivity of transrectal ultrasonography being worse than that assessment with a Metricheck® when cows were examined between 46-86 DIM. Therefore if the DIM at examination range in a herd is >45 DIM, assessment with a Metricheck® may be the more valid test. However, if most of the herd is between 25-45 DIM then transrectal ultrasonography would likely be a more useful screening test. Additionally, further research is required to consider the benefits of performing both tests 'in parallel' (see section 6.4).

6.3.5 Decisions relating to which cows to treat

Based on Chapter 3, it is fair to conclude that dairy cows with mucopurulent discharge (or ≥ score 2 on the 0-3 scale) or cows with greater than or equal to trace amounts of intrauterine fluid (i.e, or ≥ score 1 on a 0 to 4 scale) had impaired reproductive performance. However, the optimal threshold for disease positivity determined based on the Bayesian latent class analysis was ≥ score 1 for both PVD and UE scores. Although the thresholds are in agreement for UE score (i.e. ≥ score 1) they are not for PVD score (i.e. ≥ score 2 and ≥ score 1). To understand this difference, it is import to remember how these thresholds were determined. The first analysis was based on reproductive outcome in the breeding season while the second was based on the presence or absence of the target condition at the time of conducting the pre-breeding examination which are not the same. The interpretation of this then means that flecks of purulent material on the day of the pre-breeding examination indicates the presence of reproductive tract disease but this level of inflammation does not impact reproductive performance. This finding does contradict with previous work by other authors (McDougall et al., 2007; Giuliodori et al., 2017) and the same discrepancy or 'paradox' has been noted by others (McDougall et al., 2020). Findings that related to reproductive performance are the most useful to the farmer, therefore based on these findings of this research and reviewing the literature, the practical recommendations regarding which cow to treat should be as follows;

- Cows with mucopurulent discharge at the pre-breeding examine should be treated.
- Cows with greater than or equal to trace amounts of intrauterine fluid should be treated.
- Cows with fleck of purulent material and that are diagnosed with less than 3 weeks to the start of the breeding season should be considered for treatment.
- As this was an observational study and treatments were not assessed it is beyond the scope to advise on the type of treatment that should be used but commonly used treatments include intrauterine antibiotics (i.e., cephapirin) or administration of PGF2α if a corpus luteum is present and has been well reviewed by others (Lefebvre and Stock, 2012).

6.4 Recommendations for further research in relation to relating to the reproductive tract disease research

Many of the limitations of each analysis are outlined within their respective chapters, however, some are worth a further mention if further research is to be conducted in this area again prior to recommendations are outlined.

Both chapters 2 and 3 were retrospective observational cohort studies based on existing farm records, pre-breeding examinations and pregnancy results. The retrospective nature of the study meant that no adjustments to the study population or design could be made. Additionally, as it was conducted retrospectively in commercial herds, cows were treated for the reproductive tract disease when diagnosed potentially biasing some of the reproductive tract disease outcome estimates. The other disadvantage of this approach was that certain important risk factors that might contribute to the development of reproductive tract disease, notably clinical diseases such as metritis, hypocalcemia, ketosis, abomasal displacements were not accurately recorded and thus were not assessed as part of the analysis and therefore no inferences could be drawn.

The other limitation is the herds used in this study. As cows in the study were from a convenience sample of herds that were already involved in some routine herd health monitoring it is possible some of the inferences made based on these cows are not reflective of the typical Irish dairy cow and a more random sample of herds may have delivered a more representative sample where aa more representative inference could be made.

Additionally, it is worth pointing out at this point that the risk factor analysis presented in Chapter 2 were based on thresholds that were later determined to be higher than those selected as the optimal threshold for disease at time of sampling noted in Chapter 3. In Chapter 2, disease positivity was determined as greater than or equal to mucopurulent (≥ score 2) for PVD scoring and greater than a small volume of intraluminal fluid (≥ score2) for UE scoring and both were based on the results of previous research from this research group (Šavc et al., 2016). However, in Chapter 3 optimal threshold for disease at time of sampling were as greater than or equal to flecks of pus (i.e., ≥ score 1) for PVD scoring and greater than a trace volume of intraluminal fluid (i.e., ≥ score 1) for UE scoring. Consequently, the risk factors analysis presented in Chapter 2 excluded cows that had flecks of purulent material (i.e., score 1) or cows a trace volume of intraluminal fluid (i.e., score 1) and may bias the output of this analysis to cows with greater severity of disease.

With these and other considerations in mind, this thesis has identified a number of recommendations for future research as well as future research needs and they are outlined below;

- Further observational studies on commercial farms should be prospective and based on a cows from a random selection of Irish herds.

- These studies should include additional clinical conditions that should be typically recorded in commercial farms such as metritis, hypocalcemia, ketosis etc. These should be recorded using pre-defined definitions of each disease to assist in transparent and appropriate recording.
- Many studies have been conducted examining the cow-level risk factors but there is a need for more herd-level studies so better advice around hygiene and housing management can be assessed in relation to reproductive tract disease.
- There is a paucity of research in relation to of reproductive tract disease occurring in the first 3 weeks after calving (i.e., metritis and retained fetal membranes) in Ireland. There is little to no understanding of the incidence, risk factors and the farmer or veterinary approach to diagnosis and treatment and more studies in this area would be beneficial.
- Optimum thresholds for disease positivity of reproductive tract disease should be determined with similar Bayesian approach in the absence of a gold standard at the time of sampling as well as reporting the reproductive outcome in the population of cows examined in agreement with other authors (deBoer et al., 2014). Additionally, it is important optimal thresholds for the dataset analyzed are determined before any risk factor analysis is performed.
- There has been much research on visually assessment of vaginal discharge for PVD and cytological methods to assess subclinical endometritis but much less on ultrasonography and therefore further research using ultrasound as a diagnostic method in commercial herds is required.
- This research on ultrasonography should be expanded to assess the practicality, determine the optimal thresholds for positivity using a Bayesian methodology and report the associated reproductive performance.
- Studies on ultrasonography should also report on more data on intra- and interobserver agreement to evaluate test variability, something which was not assessed in this research.
- Many of the practical cow-side tests for reproductive tract disease (such as use of the Metricheck®, vaginoscopy and ultrasonography) are easy to perform and inexpensive but individually have low sensitivity and reasonable to high specificity. In the case of seasonal systems where time to intervene is limited, a diagnostic test with high sensitivity (i.e., one that result in fewer false negatives) may be more important than that of one with high specificity (i.e., one that result in fewer false negatives has fewer false positives) (deBoer et al., 2014). One method to improve diagnostic test sensitivity therefore would be to assess theses test in parallel in a practical commercial setting.
- Testing in parallel (i.e., applying two or more tests to an entire population or to initial test negative animals) may be of use in the case reproductive tract disease when using multiple inexpensive and easy to apply tests as it should result in a net gain of diagnostic sensitivity with a net loss specificity. Therefore, further studies that report multiple tests should consider how the perform when they are determined in parallel.

6.5 Main conclusion drawn from studies relating to the estrus detection inaccuracy research

The main conclusion on estrus detection inaccuracy from Chapter 4 of the thesis were:

- In a cohort of Irish milk recording dairy cows, the median overall cow-level true prevalence of inaccurate estrus detection and thus of inaccurate AIs was 4.4% (95% BPI; 1.7–9.0%).
- There was an observed lower apparent prevalence of inaccurate estrus detection and thus inaccurate AI in first serves (6.4%; 29/452) versus repeat serves (13.7%; 17/124).
- To diagnose a cows as not in estrus at AI and using a threshold of ≥5ng/ml of progesterone in whole milk and a commercial available radioimmunoassay had a median test sensitivity of 83.0% (95% BPI; 65.0–96.2%) and a specificity of 97.4% (95% BPI BPI; 94.6–99.6%).

The main conclusion on estrus detection inaccuracy from Chapter 5 of the thesis were:

- There was an observed overall apparent prevalence of inaccurate estrus detection and thus inaccurate AI in 4.7% (49/1035) of all samples serves.
- There was an observed lower apparent prevalence of inaccurate estrus detection and thus inaccurate AI in first serves (3.3%; 30/900) versus repeat serves (14.1%;19/135).
- The risk factors that were identified for estrus detection inaccuracy included;
    - At the first insemination;
        - The absence of a mounting abrasions observed on the cow at the time of AI.
    - At repeat insemination;
        - The absence of a mounting abrasions observed on the cow at the time of AI.
        - The absence of an observed standing estrus.
        - An abnormal preceding repeat interval since the last AI.

6.6 Practical implications of findings to Irish farmers and advisors relating to the estrus detection inaccuracy research

The quantification of estrus detection inaccuracy and thus inaccurate inseminations and the identification of some of the cow-level risk factors have practical implication for farmers, their advisors and AI technicians. These implications relate to following areas and are discussed below individual in relation to the conclusions;

- Identification cows that are at risk of being AI'ed when not in estrus.
- Potential strategies to implement to either prevent insemination or mitigate pregnancy in at risk cows.

6.4.1 Identification of cows that are at risk of being AI'ed when not in estrus.

Farmers and AI technicians could use some of the information generated from both studies in combination with existing knowledge and advice from the research literature to identifying cows that might have signs that indicate they at risk of being incorrectly identify as being in estrus and thus being inappropriately AI'ed. It is important to remember that both Chapter estimate prevalence and risk factors at cow-level and not at herd level. This means that the risk of a cow being inseminated will largely depend on the quality (i.e., accuracy) of estrus detection that is being conducted on a specific farm. Large difference in estrus detection accuracy between herds is well documented by some (Morton and Wynn, 2010) and did also seem to be apparent in Chapter 4 although within herd estimation of prevalence was not the research goal. Therefore knowledge of the cow-level prevalence of inaccurate AIs is not likely of large benefit to farmers as the level of it will be dependent on their own farm, however, it may be useful to stress to AI technicians who will inseminate a range of cow across a vast amount of herds. Lastly, it is should be emphasized to farmers that although an inaccurate inseminations may impact reproductive performance (notably in repeat AIs) at a cow-level, apprehension about inaccurate AI in cows should not result in a decreased probability of submission of all cows for AI as this is key for herd reproductive performance. Many authors have shown that it is possible to improve the accuracy of estrus detection without hamper estrus detection efficiency (Morton and Wyn, 2020). That stated some practical recommendations to farmer's and AI technicians originating from on the study results and a review of other literature are made below;

- Overall the current cow-level estrus detection inaccuracy appears low with about 1 in 20 cows submitted for AI being inaccurate. This was similar across two study populations.

- There also appears to be higher amounts of estrus detection inaccuracy in cows submitted for repeat AIs with about 1 in 7 cows submitted for AI being inaccurate, so greater care shown be taken with these cows. Again, this was similar across two study populations.
- Of those that are sign or signals the cow may show to indicate she is not in estrus, the absence oof mounting abrasions appears the most useful.
- Additionally, non-observation of a standing estrus event and an abnormal repeat interval (i.e., either a short period of 3-17 days versus a long period of 25-35 days) are also useful in indicating a cows is not in estrus, particularly at repeat insemination.

6.4.2 Potential strategies to implement in order to either prevent insemination or mitigate pregnancy loss in at risk cows.

The outputs of Chapter 4 and 5 did not test preventive strategies to prevent insemination or strategies to mitigate pregnancy loss. However, the following section will serve to make some recommendations that may be beneficial based on the review of the literature in this area. Some practical recommendations to farmer's and AI technicians are therefore outlined below;

- Although research is limited research (Macmillan et al. 1977), it is likely that if a first insemination is inaccurate, but it is followed by a second insemination performed when a cow is truly in estrus that the pregnancy per AI to that insemination. This means if an inaccurate first insemination is followed by a relatively short period then the overall pregnancy performance should be unaffected.
- Therefore, as the prevalence appears low and inaccurate insemination is unlikely to impact on reproductive outcome it appears the only benefit to detecting these cows is to save on wasted semen costs. This probably does not justify the use of cow side milk progesterone tests (Ingenhoff et al., 2016) across all cows at first insemination or even those cow that lack abrasions at first insemination.
- As it is more likely that repeat inseminations based on this study have a higher prevalence and that they also are more likely to impair reproductive performance (Sturman et al., 2000), much of the focus should be on cows at repeat inseminations.
- Potential options for repeat insemination are the use of cow-side lateral flow tests in milk on the day of AI (Waldmann and Raud, 2016) focusing only on cow's with the absence of mounting abrasions, an observed standing estrus event or an abnormal repeat interval and not inseminate cows with a positive test (i.e., high milk P4)
- Alternative to this approach, the farmer or the AI technician could exercise more caution in cows with these signs and that may involve alternating the insemination location to mid cervical versus intrauterine (Weaver et al., 1989) or using some form of sanitary sheet to minimize uterine contamination with bacteria (Bas et al., 2011) or both in combination.

6.7 Recommendations for further research in relation to the estrus detection inaccuracy research

Much of the work related to estrus detection inaccuracy in this thesis was preliminary work on quantification of levels of inaccuracy and identification of some risk factors that would help identify cows that might be eligible possible interventions. With these considerations in mind, this thesis has identified a number of areas for future research in these areas and it is outlined below;

- There is a real need to quantify the herd level studies on estrus detection inaccuracy. Previous work in Australia (Morton and Wynn, 2010) and to an extent in this thesis, illustrated that the within herd prevalence of estrus detection inaccuracy can be high in up to a quarter of herds and better quantification of this and developing a better understanding of why would help in providing farmer with better preventative advice around this.
- The approach of Mortan and Wynn (2010) in Australia used strategic milk sampling with progesterone to estimate level of estrus detection inaccuracy (or as they term 'the positive predictive value of ovulation') but also estrus detection efficiency in a wide range of seasonal calving herds. Therefore, if an approach to quantify herd -level variation in estrus detection inaccuracy, a concurrent assessment of estrus detection efficiency and factors related to it would be as if not more important to the Irish dairy industry.
- This thesis and literature review has also highlights the general paucity around research relating to the impacts of inaccurate AI on reproductive performance. Previous work in Ireland has potentially indirectly linked estrus detection inaccuracy to poor reproductive performance as cows with an history of abnormal repeat intervals were associated with reduced in-calf rates. Further work assessing the impact that inaccurate inseminations have on reproductive outcome in a larger sample of Irish cows in multiple herds is needed to assess the true impact.
- This study has also highlighted the lack of research related to areas such as the impact of timing in relation to a previous insemination, insemination methods and insemination hygiene have on cows who are inseminated at the incorrect time. Many of the studies in relation to timing (Macmillan et al., 1977), methods (Vandenmark et al., 1952; Weaver et al., 1989) and hygiene (no specific found) are quite dated, or no data exists.
- As an example of this from a review of literature, is that there is an assumption that if a first insemination is inaccurate, but it is followed by an accurate insemination it has been shown to have little detrimental effect on the probability of pregnancy at the following accurate insemination (Macmillan et al., 1977). However, if an unhygienic insemination introduces bacteria during the luteal phase, it is more likely to induce an iatrogenic endometritis due to the immunosuppressive effects of P4 and this has the potential to impact the future probability of pregnancy at AI (Pascottini et al., 2017). Further research in this area would be interesting.
- This thesis has suggested using the risk factors identified to help target cows for the use of cow-side milk P4 to confirm whether the cow is indeed not in estrus. As already mentioned in

relation to reproductive tract disease, this approach constitutes test ‘in series’ type approach to the condition but this was not tested in this thesis using cow-side test like the lateral flow and further research should be conducted in the field to test if it is an appropriate approach that makes economic sense.

- Likewise other strategies such as altering the location of AI or the use of plastic sheets to improve hygiene on repeat inseminations to mitigate the negative impacts of an inaccurate AI in repeat insemination when a cow is potentially where not tested in the field and should be conducted to test this hypothesis.

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