PREDICTING OESTRUS AND OVULATION IN SOWS USING NOVEL PHYSIOLOGICAL MARKERS

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PhD THESIS

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Declaration

I declare that this thesis contains original research written by the author that has not previously been submitted for fulfilment of any other degree or at any other institution.

I provide permission for this thesis to be made available in a digital format that enables access to this research online.

Dannielle Glencorse

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Abstract

Detection of oestrus in pigs occurs predominantly through monitoring for the presence of standing heat, the behaviour change indicative of sexual receptivity. Standing heat can be used to identify the oestrus period but is not capable of precisely identifying ovulation and the optimum timing for insemination. As a result, breeding protocols implement double inseminations repeated over the 2-3 day oestrus period to ensure viable spermatozoa are present within the reproductive tract at ovulation. Multiple inseminations result in wasted semen doses and high labour inputs which could be prevented with the development of an accurate oestrus detection tool.

This project is focused on investigating and evaluating novel markers that enable rapid identification of ovulation and the optimum time for mating with high precision. There are several physiological changes in sows that could be used as alternative markers. This research focuses on several markers during oestrus; electrical resistance of the reproductive tract, cervical mucus composition, body temperature and quantified behaviour monitoring.

Oestrus was clearly distinguished through observation of elevated activity levels and distinctive signal profiles, detected using collar-mounted accelerometers. This technology is a precise potential replacement of existing oestrus detection protocols which could reduce daily labour requirements for this task. Ovulation occurred simultaneously with sharp increases in vaginal electrical resistance (ER), a reduction in cervical mucus pH and sodium levels, increased likelihood of linear cervical mucus patterns and increased vulva temperature. The recommendation from the current study is to conduct inseminations immediately upon observation of any of these markers. Individual markers can enable accurate detection of ovulation status. However, if multiple markers are identified, a more productive and efficient conception outcome is likely leading to more profitable sow management.

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Chapter 1

Literature Review

1.1 Introduction

An efficiently produced food supply is essential with the current exponential rates of global population growth (Kendall & Pimentel 1994). Agricultural industries can provide meat to the growing population by rearing livestock for slaughter (Godfray et al. 2010). The supply of meat for human food consumption required approximately 60 billion animals in 2010 (Prakash & Stigler 2012). Meat consumption has continued to grow with recent estimations indicating daily meat consumption of 122 grams per person, leading to a global requirement for over 300 million tonnes of meat p.a. (Godfray et al. 2018). The number of individual animals required to sustain the demand for meat is growing, leading to industries that are focusing on intensive farming instead of the small herd sizes typical of agricultural farming in the twentieth century (Tilman et al. 2002). Animal industries involving species such as pigs (*Sus scrofa)* and chickens *(Gallus gallus)* are more capable of large-scale, sustainable production as they are predominantly intensive systems and can efficiently produce higher quantities of meat than traditional extensive red meat industries (Tilman et al. 2002). The average herd size of pig breeding farms in Australia was 170 sows in 2013 and was recorded at 170-180 in 2016 (APL 2013; IBISWorld 2016). Over 65% of these animals were farmed in herds of more than 1000 breeding sows (APL 2013). These larger herd sizes introduce issues with animal management, predominantly due to the increased number of animals that are cared for by each stock-person (Babot et al. 2003). The increase in herd size required to enable adequate supply leads to the need for new processes that are less labour intensive and time consuming (Gardner et al. 2002).

One way to account for the increased labour demand from less staff members is to introduce new tools, techniques and technologies to quantify, objectify and automate intensive farm practices (Kashiha et al. 2014). One production area that would benefit from more objective protocols is the management of breeding and reproduction. Sow reproduction is complex as the process is affected by multiple factors and results are variable within and between farms (Plà 2007). Reproductive success is crucial for continued animal production. Conception rates vary depending on environment, management practices, animal factors and stock-person skill and is therefore a process that would benefit from the application of novel

techniques (Knox 2016). This is evident in the process of detecting oestrus and determining the timing of inseminations (Weitze et al. 1994). Detection of sexual receptivity, otherwise known as oestrus, involves observation of oestrus behaviours which dictates when sows are inseminated (de Jonge et al. 1994). This is a subjective process that requires significant labour input and staff training to ensure accuracy (Cornou 2006). This part of the breeding process is ideal for novel techniques as improvement in the accuracy and precision of the current oestrus detection methods would enhance the overall productivity and profitability of pig farming (Banhazi & Black 2009).

1.2 Artificial insemination

Within the pig industry, artificial insemination (AI) is used frequently due to numerous advantages in comparison to natural mating (Knox 2011). Artificial insemination is better able to disseminate the genetics of boars with superior qualities to obtain the higher levels of production (Bailey et al. 2008; Didion et al. 2013; Men et al. 2012). Purchasing individual semen doses with desirable breeding capacities is also significantly cheaper than acquiring and caring for boars on farm (Gadea 2003). By using AI, producers can avoid the continuous use of a single genetic line from one boar which can lead to inbreeding and the transfer of recessive non-lethal and non-desirable disorders through the herd (Holt 2000; Knox 2011). In order to conduct an AI program, accurate oestrus detection methods need to be implemented (Belstra et al. 2004). It is vital that inseminations occur at the appropriate time to ensure spermatozoa are present in the oviduct as the oocytes are released from the ovaries (Steverink et al. 1998). The optimum timing of inseminations depends on the lifespan of spermatozoa, which is different when using either liquid-stored or frozen-thawed spermatozoa (Rath et al. 2009).

1.2.1 Liquid-stored artificial insemination

Liquid stored AI is the most commonly used practice in the pork industry and accounts for over 70% of inseminations conducted on Australian pig farms and 90% of inseminations worldwide (Didion et al. 2013; Knox 2016). The successful use of AI with liquid-stored semen within the Australian pig industry has improved the profitability of individual producers and supported the expansion of Australian pig numbers to allow for access into global markets

(Bailey et al. 2008; Knox 2011). The longevity of liquid-stored semen doses is optimised for 3-7 days post collection by diluting and storing at 16-17°C (Roca et al. 2006b). A post-collection lifespan of up to 10 days is possible although this coincides with a small reduction in motility to 75% (Becherer et al. 2014). Widespread distribution of semen throughout Australia is possible using liquid-stored spermatozoa as chilled samples are easily transported in an adapted refrigerator (Didion et al. 2013; Men et al. 2012). The conventional protocol requires 2-3x10^9 individual spermatozoa per insemination dose, which yields farrowing rates of 80-90% and more than 10 piglets per litter, which is comparable to the fertility of natural mating (Cassar et al. 2011; Roca et al. 2011). In order for liquid-stored spermatozoa to survive until fertilisation and maximize farrowing rates, insemination must occur within 24 hours of ovulation (Rath et al. 2008). While the use of liquid-stored semen is highly successful, there is much interest in developing methods for use of cryopreserved spermatozoa in AI programs (Didion et al. 2013; Knox 2011; Ringwelski et al. 2013).

1.2.2 Frozen-thawed artificial insemination

The use of frozen-thawed artificial insemination (FTAI) has several benefits when compared with chilled spermatozoa (Grossfeld et al. 2008). These include the ability to transport globally, long-term storage and conservation of superior genetics over longer periods of time (Bailey et al. 2008; Didion et al. 2013; Knox 2011). Despite these advantages, only 1% of all AI procedures within the Australian pork industry use frozen-thawed spermatozoa (Ramos 2011; Wagner & Thibier 2000). There are several reasons for the limited implementation of FTAI programs into global pork production. One factor that contributes to poorer AI outcomes, particularly with frozen-thawed spermatozoa is the imprecise oestrus detection methods that are used to identify the optimum time for insemination (Řezáč & Olic 1988; Roca et al. 2011). A range of other drawbacks exist including lowering of farrowing rates by approximately 40%, and extensive costs associated with semen processing (Broekhuijse et al. 2011; Casas et al. 2010; Didion et al. 2013; Grossfeld et al. 2008). The primary reason for poor utilisation rates of FTAI is based on the extensive labour requirements that are necessary to ensure that oestrus is detected accurately and that the timing of inseminations is optimum (Hühn et al. 1996; Knox 2016). The cryopreservation and thawing processes involved in FTAI result in a reduction in spermatozoa motility and DNA integrity meaning that the lifespan of

these cells is reduced to 4-8 hours (Bathgate 2011; Fraser et al. 2011; Lewis & Aitken 2005; Waberski et al. 1994). Recent research has indicated that conception rates comparable to liquid-stored AI are obtainable in scientific trials when insemination is performed within 16-24 hours of ovulation (Bolarín et al. 2006; Eriksson et al. 2002; Martinez et al. 2002; Roca et al. 2003; Waberski et al. 1994; Wongtawan et al. 2006;). However, the translation of these results to the commercial setting has seen less favourable results as perceived by producers (Knox 2016). While this is a significant issue, these commercial results could be related to management and therefore optimum oestrus detection would reduce the severity of this issue (Watson & Behan 2002).

1.2.3 Timing of artificial insemination

There are varied AI schedules which are used in commercial processes. These programs differ in areas such as method of semen storage, location of inseminate deposition, dose rate, number of inseminations and the timing of AI (Knox 2015). While the execution of AI is often varied, these programs have one common aim; to deposit spermatozoa into the reproductive tract within close proximity to the time of ovulation in order to maximise the chance of fertilisation (Rodriguez-Martinez 2007). A typical breeding program involves two doses of semen, with a dose rate of approximately 2.5-3 x 10^9 spermatozoa deposited in the cervix following the first observed instance of behavioural oestrus (Broekhuijse et al. 2015; Soede et al. 1995). However, a range of breeding programs have been investigated with varying degrees of success (Table 1.1).

The timing used for inseminations is diverse, as it is based on detection of signs of oestrus. It is a responsibility which is assigned to stock-people with a range of skill levels (Coleman et al. 2000). The consensus from previous studies (summarised in Table 1.1) appears to be that the optimal insemination window is between 28 hours prior to ovulation to the time of ovulation. This ensures maximal sperm retention in the oviduct thus ensuring that all oocytes are fertilised, whilst allowing for variation in ovulation timing between sows (Luño et al. 2012). However, the myriad of studies into timing of insemination rely heavily on accurate and precise oestrus detection which is a major limitation in a commercial setting. Waberski et al. (1994) found that single inseminations more than 8-12 hours prior to ovulation resulted in a 41%

decrease in farrowing rate while Bortolozzo et al. (2005) expanded the range to 16 hours without a reduction in conception. Other studies found that the threshold for successful AI within the 24-hour period prior to ovulation is uncertain (Vazquez et al. 2005). Several studies have indicated with confidence that inseminations that occur more than 24 hours prior to ovulation result in severe reductions in farrowing rate (Bortolozzo et al. 2005; Kemp & Soede 1995; Nissen et al. 1997; Soede et al. 1995). This result is amplified when using FTAI programs where the lifespan of spermatozoa is further reduced to 4-8 hours (Althouse & Lu 2005; Waberski et al. 1994; Wongtawan et al. 2006). This knowledge has been used when designing commercial AI programs which use double or triple inseminations (Soede et al. 1995). Multiple dose AI programs are commonplace within the industry and usually involve two or three doses administered approximately 24 hours apart (Spencer et al. 2010). This practice ensures that viable spermatozoa are always present within the reproductive tract during the oestrus event, so there is less reliance on accurately detecting ovulation and the associated optimum time for insemination (Rodriguez-Martinez 2007). However, there is double the labour requirements when conducting AI several times for each animal (Flowers & Alhusen 1992). Similarly, sows are retained in mating facilities for longer lengths of time which therefore impacts on management of space allocations (Flowers & Alhusen 1992). Single insemination AI programs overcome these concerns but are rare as precise deposition of spermatozoa is difficult when using subjective and variable behaviour-based oestrus detection (Knox 2014).

A unique approach to overcoming the limitations of single AI programs is to implement detection protocols which focus on identifying physiological changes associated with ovulation rather than focusing on the broad period of oestrus (Firk et al. 2002; Hunter 1977; Senger 1994). While oestrus length is inconsistent between sows, the timing of ovulation is relatively consistent (Belstra et al. 2004; Nissen et al. 1997), occurring approximately 70% of the way through behavioural oestrus in both sows and gilts (Bortolozzo et al. 2005; Roca et al. 2011). There has been difficulty in using this information in commercial settings due to variation in oestrus length between individual sows, a factor that can only be measured retrospectively (Steverink et al. 1999). As a result, ensuring that AI is conducted to coincide with ovulation is very difficult. To maximise the farrowing rate of commercial herds, stock-

people should focus on attaining an understanding of the oestrus period and conduct detection programs to identify physiological markers of ovulation.

Table 1.1 Fertility rates obtainable using different semen and insemination types, dose rates and number of inseminations.

Semen type	Site of deposition	Number of sperm/dose	Number of inseminations	Insemination timing relative to ovulation	Farrowing rate (%)	Reference
Liquid-stored	Cervical	2 x 10^9	Single	16-12 h prior	55.8%	Waberski et al. 1994
				12-8 h prior	96.0%	
				8-4 h prior	85.5%	
				4-0 h prior	93.4%	
				0-4 h after	92.5%	
				4-8 h after	71.4%	
				8-12 h after	58.9%	
Liquid-stored	Cervical	Not reported	Double (4-hour interval between AI)	22 h prior	46.7%	Ringwelski et al. 2013
				10 h prior	73.7%	
				2 h after	85.3%	
				14 h after	55.1%	
Liquid-stored	Cervical	4 x 10^9	Single	>32 h prior	77.8%	Bortolozzo et al. 2005
				24-32 h prior	70%	
				16-24 h prior	88.9%	
				8-16 h prior	100%	
				0-8 h prior	100%	
Liquid-stored	Cervical	2 x 10^9	Single	36-29 h prior	50%	Nissen et al. 1997
				28-25 h prior	89%	
				24- h prior	88%	
				0-4 h after	83%	
				5-9 h after	75%	
Liquid-stored	Cervical	3 x 10^9	Single	>24 h prior	55%*	Kemp & Soede 1995
				0-24 h prior	91%*	
				0-16 h after	71%*	
Liquid-stored	Cervical	3 x 10^9	Single	>48 h prior	35%	Soede et al. 1995
				48-40 h prior	51 ± 36%	
				40-32 h prior	54 ± 36%	
				32-24 h prior	79 ± 32%	
				24-16 h prior	94 ± 11%	
				16-8 h prior	92 ± 21%	
				8-0 h prior	95 ± 22%	
				0-8 h after	75 ± 38%	
				8-16 h after	74 ± 43%	
				>16 h after	0%	

Semen type	Site of deposition	Number of sperm/dose	Number of inseminations	Insemination timing relative to ovulation	Farrowing rate (%)	Reference
Liquid-stored	Cervical	1 x 10^9	Single	12-24 h prior	89 ± 15%*	Steverink et al. 1997
		3 x 10^9		12-24 h prior	88 ± 28%*	
				24-36 h prior	83 ± 22%*	
		6 x 10^9		24-36 h prior	83 ± 26%*	
Liquid-stored	Trans-cervical	1.25 x 10^9	Single	24 h prior	62.8%	Garcia et al. 2007
				6 h prior	71.1%	
		2.5 x 10^9		24 h prior	79.6%	
				6 h prior	84.4%	
Frozen thawed	Cervical	5 x 10^9	Single	>8 h prior	0%	Waberski et al. 1994
				8-4 h prior	54.9%	
				4-0 h prior	88.1%	
				0.4 h after	50.0%	
Frozen-thawed & liquid-stored	Cervical	3 x 10^9	Single	12 h prior (LS)	68.8%	Abad et al. 2007
				12 h prior (FT)	10.0%	
				2 h prior (FT)	14.7%	
Frozen-thawed & liquid-stored	DUI	3 x 10^9	Single	12 h prior (LS)	92.0%	Abad et al. 2007
				12 h prior (FT)	41.7%	
				2 h prior (FT)	38.5%	

* normal embryo percentage (%)

1.3 The oestrous cycle of sows

1.3.1 Reproductive cyclicity

The oestrous cycle of a sow is 18-21 days in length and is split into two stages; the follicular phase and the luteal phase (Edström 2009; Roca et al. 2006a). The follicular phase is a period dominated by oestrogen where follicles develop in preparation for ovulation (Soede et al. 2011). The luteal phase is the period where the reproductive tract prepares for a potential pregnancy under the influence of progesterone (Knox 2011). Sows undergo poly-oestrus cyclicity with continuous oestrous cycles throughout the entire year, regardless of season (Şandru et al. 2009).

1.3.2 Oestrus and ovulation

Oestrus is a period otherwise known as heat, that occurs during the follicular phase when sows display sexual receptivity (Soede & Kemp 1997). Sows have an oestrus length of approximately 48-72 hours during which females display receptivity through expression of sexual behaviour and undergo physiological changes, particularly to the reproductive tract (Pedersen 2007; Weitze et al. 1994). The length of oestrus varies among sows and can range from 2 days in gilts to 3-5 days in mature sows (Poleze et al. 2006). Figure 1.1 demonstrates that sows undergo a gradual increase in sexual receptivity throughout oestrus which is caused by secretion of oestrogen from the ovaries (Esbenshade et al. 1990). This is caused by the hypothalamic-pituitary gonadal axis which controls production of gonadotropin releasing hormone (GnRH) in the hypothalamus (Haussmann et al. 2000). As a result of GnRH secretion, the pituitary produces and secretes follicle stimulating hormone (FSH) and luteinising hormone (LH), both of which are detected by receptors in the ovarian follicular cells (Nett et al. 2002). These hormones stimulate the production of oestrogen in the developing follicles at the beginning of the follicular phase, causing physiological and behavioural changes (Soede et al. 1997a). During the mid to late stages of the follicular phase, the concentration of oestrogen increases and triggers a positive feedback response (Soede et al. 2011). This causes a surge in the release of GnRH, triggering a subsequent surge of LH which results in ovulation 36-42 hours after the initial GnRH release (Cassar et al. 2005; Yilma & Sobiraj 2011). Ovulation is

the release of oocytes from the ovary followed by transit to the oviduct in preparation for fertilisation (Nett et al. 2002). This occurs approximately two-thirds of the way through oestrus and lasts for approximately 1-3 hours, with multiple antral stage oocytes being released (Soede et al. 1992). Ovulation of the first oocyte occurs approximately 65-70% of the duration of oestrus and subsequent ovulations continue for 1.8 ± 0.6 hours in naturally cycling sows (Soede et al. 1992). While ovulation timing occurs relatively consistently at 65-70% of the cycle across all sows (Kemp & Soede 1995; Weitze et al. 1994), there is considerable variation in the length of oestrus which means that the correct timing of ovulation can only be pinpointed retrospectively (Yilma & Sobiraj 2012). There are no current procedures which utilise this knowledge to enhance the accuracy of insemination timing (Rozeboom et al. 2004). This is because variation in oestrus length means that the time of ovulation is only known to the producer retrospectively (Belstra et al. 2004). As a result, variation in oestrus length is one of the main factors that have hindered producers from identifying the optimum insemination timing (Cassar et al. 2005).

Oestrus length varies due to differing intervals between the onset of behavioural oestrus and ovulation, otherwise known as the oestrus-ovulation interval as well as the weaning-oestrus interval (Soede & Kemp 1997; Steverink 1999). These intervals fluctuate based on several factors including age, with gilts having a significantly shorter period (Belstra et al. 2004; Gesing 2015), season (Love et al. 1993), parity (Tantasuparuk et al. 2000) and genotype (Rydhmer 2000). In addition, the period of ovulation can last up to 5 hours, hence adjusting the total length of oestrus (Soede et al. 1997a). As oestrus duration is relevant for determining when ovulation occurs and thus the optimum time for insemination, it would be beneficial to predict the duration (Soede & Kemp 1997). There has not been any observed correlation between the concentration of oestrogen and the length of observed behavioural oestrus (Andersson et al. 1984; Soede et al. 1994). This indicates that oestrogen has an impact on the changes to behaviour but does not support the hypothesis that the oestrus length is dictated by the concentration of oestrogen (Pedersen 2007).

Oestrus detection is a multi-factorial relationship and as such, requires more efficient tools to accurately predict the optimum time of insemination. An objective examination of the

physical and behavioural changes that are present during oestrus could identify markers to improve the precision of oestrus detection (Belstra et al. 2004).

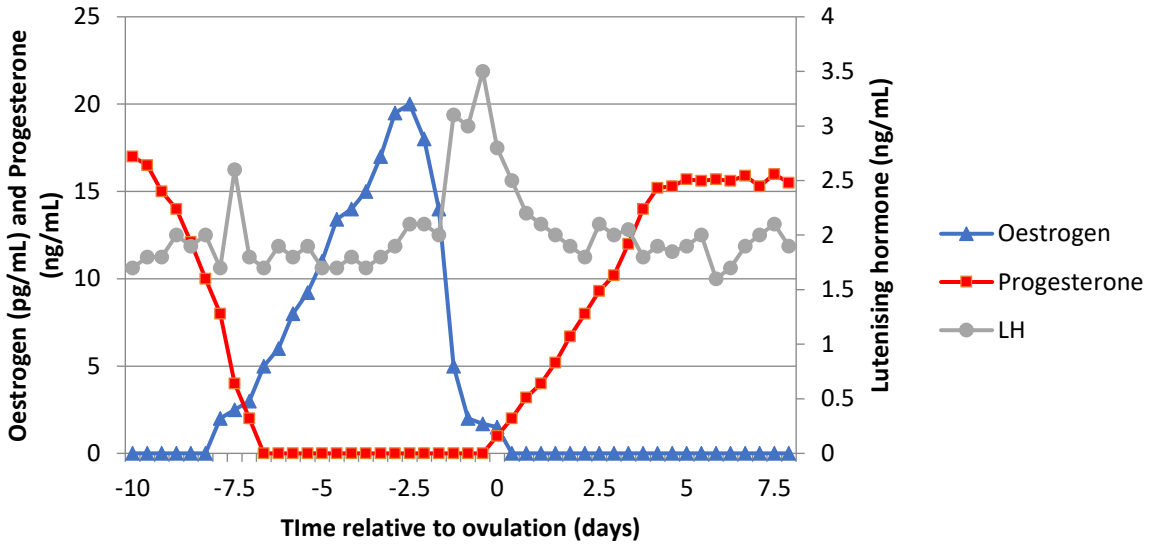

Figure 1.1 Representative schematic displaying the hormonal profiles of oestrogen, progesterone and luteinising hormone that occur during the oestrous cycle of sows (adapted from Soede et al. 2011).

1.3.3 Physical changes

The reproductive tract undergoes substantial physical changes caused by secretion of oestrogen from the developing follicles in the sows' ovaries (Soede et al. 2011). The epithelial lining of the female reproductive tract contains receptors which detect oestrogen in elevated concentrations in the early stages of oestrus (Ježková et al. 2008; Yilma & Sobiraj 2012). These changes occur to facilitate a suitable environment that will support spermatozoa transit and survival during fertilisation (Foxcroft & Hunter 1985). Oestrogen secretion causes physiological changes in the reproductive tract that includes an increase in cervical mucus production, not only in terms of quantity but also consistency (Betteridge & Raeside 1962). Cervical mucus becomes thicker, denser and smoother during oestrus due to the presence of dissolved substances such as sodium, chloride and bicarbonate ions and protein-based mucins (Luño et al. 2012). The consistency changes because of the ratio of sodium and chloride ions

present within the secretion, with a larger concentration of sodium occurring at ovulation (McSweeney & Sbarra 1964). Other changes include oedema and hyperaemia of the vulva and epithelial linings of the reproductive tract (Ježková et al. 2008; Yamauchi et al. 2009; Yilma & Sobiraj 2012). The uterine secretions function by providing lubrication for the tract, acting as a medium for sperm transport, causing contractions in the epithelial lining and reducing immune activity by retaining leucocytes in sub-epithelial layers which make it conducive to sperm movement (Bortolozzo et al. 2005). These physiological effects occur from the onset of the follicular phase until 2-3 days after the cessation of oestrus (Yilma & Sobiraj 2012). However, the intensity of each of these changes varies through the oestrus period which may be adjusted by oestrogen secretion (Luño et al. 2012). Additionally, the severity of these physiological changes varies between sows as the concentration of oestrogen often differs from animal to animal (Ringwelski et al. 2013).

1.3.4 Behavioural changes

Animal behaviour changes with the purpose of indicating the status of an individual (Ringgenberg et al. 2010). Sexual behaviour is controlled by the nervous system with neurotransmitter-based hormonal signals released from the hypothalamus (Soede et al. 1997a). During oestrus, the purpose of sexual behaviour is to enable the sow to communicate sexual receptivity and encourage the opportunity for copulation (Signoret 1970). Behaviours that occur during oestrus have several roles: attraction of the male attention, initiation of copulation and receptivity during the sexual congress (Pedersen 2007).

Three classifications of behaviours associated with oestrus have been described; attractive, proceptive and receptive behaviours (Hemsworth 1985; Pedersen 2007). Attractive behaviours are often large, obvious signals that can be distinguished from a distance (de Jonge et al. 1994). These actions which aim to attract attention of potential mates are repetitive and may involve other animals with examples including posture changes, elevated activity levels and vocalisation (Hemsworth & Tilbrook 2007). Proceptive or appetitive behaviours occur to induce sexual activity during proestrus and prior to standing heat (Beach 1976; Pedersen 2007). During this time, sows will undergo behaviours such as female to female mounting, nudging, smelling or licking the urogenital area of other sows, flank stimulation, pursuing other sows or

boars, human interaction, and snout contact with other sows (Pedersen 2007; Soede et al. 2011). These behaviours become more frequent and apparent in response to visual, auditory, olfactory and tactile stimuli from the male and as such, producers use the physical presence of the boar to improve oestrus detection rates (Blair et al. 1994; Hemsworth 1985). Receptive behaviours are those that demonstrate acceptance of copulation (Langendijk et al. 2003). They occur when the sow undergoes mating or insemination and are usually determined by observing the presence of standing heat, an adjusted posture with immobility that enables the sow to accommodate for the boar's weight (Weitze et al. 1994). Standing heat or standing oestrus can be identified by using the back-pressure test to determine if the sow is displaying a rigid lordosis posture with the spine arching ventrally, squared limbs, raised hips and will minimise excessive activity to allow successful copulation to occur (Hemsworth & Tilbrook 2007). The back-pressure test is the most commonly used method for detecting the receptivity of sows in commercial production as the receptive behaviour occurs in response to physical stimulation by boars or stock people (Pedersen et al. 2003). The timing of receptive behaviour is beneficial as it occurs during the middle of the oestrus period and relatively close to ovulation (de Jonge et al. 1994; Špinka 2009). However, the intensity of standing heat varies between individual sows, with some displaying minimal or no changes in posture, often making the process dependent on the ability and experience of stock-people (Bressers et al. 1991). Some animals do not display any behavioural change whilst still undergoing the hormonal fluctuations that define oestrus (Soede et al. 1997a; Špinka 2009). Similarly, sows and gilts present varied oestrus behaviours, with the later more likely to demonstrate clear boar seeking and standing heat behaviours (Rodrigues 2018). High staff competency is necessary to ensure that this behaviour is detected, particularly as subjectivity is often an issue with inexperienced staff (de Jonge et al. 1994).

1.4 Commercial oestrus detection procedures

Standard insemination protocols involve a double insemination occurring 12-24 hours apart, beginning after detection of standing heat (Hunter 1977). Producers must be able to reliably determine the presence of oestrus and sexual receptivity in sows to ensure insemination occurs close to the time of ovulation to facilitate high fertilisation rates (Roca et al. 2006a).

This is because the expected lifespan of spermatozoa ranges from 4-8 hours post-thaw for frozen-thawed spermatozoa and up to 24 hours for liquid-stored spermatozoa, both of which must enter the sows' reproductive tract with sufficient time to fertilise a viable oocyte (Casas et al. 2010; Didion et al. 2013). Traditional oestrus detection requires observation of behavioural and physiological changes (Roca et al. 2006). Commercial oestrus detection protocols rely on visual observation of sows once or twice daily to subjectively assess animal behaviour and identify sows that are exhibiting sexual behaviours indicative of oestrus (Knox 2011). Accurate identification of this period of sexual receptivity is necessary to maximise the chance of pregnancy and subsequently high farrowing rates and large litter sizes (Yilma & Sobiraj 2012). The difficulty in detecting oestrus results from the large variability in individual hormonal responses and the subsequent variation in detectable sexual behaviour (Behan & Watson 2005). Variation in oestrus detection efficiency occurs predominantly as a result of differing oestrus lengths and inaccurate ovulation markers (Pedersen 2007; Perry et al. 1980).

1.4.1 Standing heat

The major focus of oestrus detection protocols on farm is the standing heat test which involves applying pressure to the back of the sow to identify lordosis (Soede et al. 2011). While the standing response can be detected by applying pressure to the back of the animal in the sows' pen, accurate detection of oestrus independent of boar presence is limited to 59% (Hemsworth & Tilbrook 2007). Many producers use boar exposure to stimulate behavioural oestrus which increases the accuracy of detection to 90-100% (Hemsworth & Tilbrook 2007). Urine, preputial fluid and saliva from boars contain the pheromone 3-α androsterol which induces sexual receptivity in sows and gilts (Pedersen 2007). The presence of boars during oestrus detection and mating also results in oxytocin release which increases the myometrial contractions that assist with movement of spermatozoa to the oviduct (Langendijk et al. 2005; Pedersen 2007). This procedure is the most common method for oestrus detection in commercial production, but it is often observed alongside several other behaviours to confirm the presence of oestrus accurately (Belstra et al. 2004).

1.4.2 Other sexual behaviours

In addition to detecting standing heat, producers will identify several other behaviours which are indicative of oestrus and initiated by the secretion of oestrogen (Pedersen 2007). These behaviours are often easier to detect if sows are housed in social groups and involve sow-to-sow interactions (Spoolder et al. 2009). The behaviours detected by stock-people include female-to-female mounting, snout contact, nose-to-urogenital interactions, flank stimulation, pursuing other sows and approaching both humans and boars (Belstra et al. 2004; de Jonge et al. 1994; Langendijk et al. 2005). These behaviours are more difficult to identify in sows that are housed individually (Freson et al. 1998). Other non-social behaviours include increased vocalisation and increased activity levels (Cornou et al. 2006; Soede et al. 1997a). While all of these behaviours are associated with oestrus, there is inherent variability between animals and therefore not all sows will display all of these signals (Hemsworth et al. 2015; Tilton et al. 1982). To ensure that oestrus can be detected in these sows despite the lack of behaviour signals, an alternate marker for the timing of insemination is required (Glencorse et al. 2017).

1.4.3 Vulva swelling

Oestrus detection procedures also include observation of physical changes to bodily processes that occur as a result of increased oestrogen concentration (Ash & Heap 1975; Sterning et al. 1994). Most notably, the vulva undergoes a series of changes including swelling and reddening because of increased blood flow to the area (Sterning 1995). This is an obvious and simple way to detect change that occurs concurrently with behavioural oestrus (Scolari et al. 2011). This technique is often used in addition to the observation of behavioural changes (Miedema 2009). However, the detection of these changes is subjective and often neglected in the training of stock-people (Willemse & Boender 1966). Vulva swelling varies based on parity and is less evident in older sows that have undergoing more parturition events (Miedema 2009). Each farrowing causes increased capillary damage and inhibits blood flow to the vulva during subsequent oestrus events (Schmidt et al. 2013)

1.4.4 Ear flicking

The muscular contractions that occur within the uterine walls to facilitate spermatozoa transit may be linked to the behaviour of ear flicking (Knox 2016). These contractions are required during the standing heat response which is present during early-to-mid oestrus (Pedersen & Jensen 1989). It is hypothesised that these contractions could extend to the rest of the body, causing the ears to form a rigid position and the ear undergoes a quivering motion. This physical change is assumed to be involuntary, only associated with oestrus and is observed alongside standing heat, but this has not been examined in detail relative to ovulation (Soede et al. 1997a).

1.4.5 Decision-making for insemination protocols

Oestrus detection through behavioural assessment will only successfully indicate the onset of the oestrus period but cannot identify the timing of ovulation and therefore the optimum time for insemination (Belstra et al. 2004). It is important to inseminate sows as close as possible to the time of ovulation as this increases the rates of fertilisation and farrowing (Bolarín et al. 2006). However, it is difficult to pinpoint the timing of ovulation within the oestrus period because of variations in individual oestrus length and behaviours displayed and the use of minimal observation and the labour required (Soede et al. 1997a). As ovulation cannot be successfully identified in real-time, producers typically rely on oestrus detection once per day, followed by repeated inseminations every 24 hours after the first detected behavioural oestrus (Steverink et al. 1999). Conventional oestrus detection coupled with a double or triple insemination regime is used to increase the chances of fertilisation as it ensures that viable spermatozoa are present in the tract when the ova are ovulated (Spencer et al. 2010). Repeated inseminations are necessary if using liquid-stored spermatozoa as the lifespan is reduced to a maximum of 24 hours post insemination (Knox 2011; Ringwelski et al. 2013; Roca et al. 2011). However, this process is time consuming, labour intensive and wastes semen (Roca et al. 2011). As a result, a more efficient method of improving productivity would involve clarification of an optimal insemination time that would maintain optimal fertility and fecundity with a single insemination (Broekhuijse et al. 2011).

1.5 Alternative oestrus detection tools

The method for detecting oestrus described above is subjective, and hence the outcomes of inseminations that use this technique are often variable (Knox 2016). To improve the efficiency of the current behavioural oestrus detection method, an accurate predictor of ovulation needs to be developed (Kemp et al. 1998). Any markers for predicting optimum insemination timing should be highly accurate with low false positive identification in a large proportion of the animals in order to be considered a valuable alternative to behaviour-based methods (Hockey et al. 2010). An ideal marker would involve a physical change that can be detected in the 24 hours prior to ovulation to ensure there is sufficient time available to plan and undertake insemination and ensure spermatozoa are situated in the oviduct for fertilisation (Řezáč & Olic 1988). This means that a suitable biological marker would require a consistent, precise and significant change in the 24-hour period prior to ovulation. This marker also needs to be consistently present in a large majority of, if not all sows (Aboul-Ela et al. 1983). The use of novel techniques and technologies that are automated, quantifiable and objective would reduce labour requirements and increase the detectability of oestrus (Fisher et al. 2008; Rutten et al. 2013). There are several existing physiological and behavioural markers that may be exploited including mucus composition, reproductive tract conductivity, body temperature and activity levels (Cornou & Lundbye-Christensen 2008; Luño et al. 2012; Scolari et al. 2011; Yamauchi et al. 2009). While these markers have been investigated individually in other studies, there has not been a study that combines these observations to determine a multifactorial method for predicting the optimum time for insemination.

1.5.1 Cervical mucus

Cervical mucus is a dense liquid produced within crypts in cervical tissue (Yilma & Sobiraj 2012) consisting of water, dissolved ions including sodium, phosphate and chloride, salts and proteins, which make up the soluble fraction. Glycoproteins or mucins make up the insoluble component (Luño et al. 2013).

1.5.1.1 Composition

The mucus composition is often considered to be dynamic as it changes rapidly during oestrus (Lai et al. 2009). Mucus is rarely homogenous, as the composition varies based on the location within the reproductive tract, with altered concentrations of soluble ions and proteins being produced and released in different sections (Edström 2009; Haynes 1971; Ježková et al. 2008; Luño et al. 2012). Soluble components such as sodium chloride ions, bicarbonate, phosphates and proteins are present in cervical mucus in addition to insoluble glycoproteins called mucins (Luño et al. 2012). In addition, the mucus composition is temporally variable with hormone concentrations dictating the quantity and structure of mucus with the changes taking time to affect the entire mucus volume (Lee et al. 2013). As the concentration of oestrogen steadily increases during oestrus, the mucosal glands regulate the quantities of the glycoprotein and ions accordingly in order to provide a medium for sperm transit following insemination (Chretien et al. 1973; Ježková et al. 2008; Luño et al. 2012).

During proestrus and early oestrus, the main component of secreted mucus is glycoproteins which predominantly consist of sialic acid (Pluta et al. 2012; Rath et al. 2008). This is significant for movement of spermatozoa which is reduced due to the dense and highly viscous nature of these molecules (Ježková et al., 2008). This type of mucus is predominant until just prior to signs of behavioural oestrus (Branscheid & Holtz 1988). This is observed as an accumulation of mucus secreted from the vulva and is associated with the onset of behavioural oestrus (Betteridge & Raeside 1962).

1.5.1.2 Volume

In addition to the modified composition, oestrogen also causes an elevated volume of mucus within the reproductive tract leading to hydration of the epithelial tissue (Řezáč et al. 2003b; Yilma & Sobiraj 2012). This is due to an increase in the quantity of active mucus secreting cells found in the epithelium of the vagina that occurs during pro-oestrus and oestrus (Abusineina 1962).

1.5.1.3 Crystallisation

Crystallisation, otherwise referred to as arborisation, refers to a visual phenomenon that involves the formation of patterns in air-dried samples of cervical mucus (Ježková et al. 2008). This has been observed in saliva and cervical mucus in several species including humans (Gruberova et al. 2006), dogs (Pardo-Carmona et al. 2010), cattle (Abusineina 1962; Ježková et al. 2008) and pigs (Betteridge & Raeside 1962; Luño et al. 2012). The increased sodium chloride and phosphate salts within the mucus accumulate around the insoluble mucins and this is referred to as crystallisation (Abusineina 1962; Betteridge & Raeside 1962; Haynes 1971). These physiological modifications cause the formation of crystalline, fern-like patterns and can be observed in a mucus smear under a light or compound microscope (Betteridge & Raeside 1962; Luño et al. 2012).

Detection of fern-like crystallisation patterns in cervical mucus has been associated with the oestrus period in sows and cows (Betteridge & Raeside 1962; Yilma & Sobiraj 2011; Yilma & Sobiraj 2012). Due to the relationship between crystallisation and elevated oestrogen levels, it has been suggested that the stage of oestrus and optimal insemination time could be determined by monitoring mucus samples (Aboul-Ela et al. 1983; Abusineina 1962).

Zink & Diehl (1984) demonstrated that the concentration of electrolytes increases prior to the onset of behavioural oestrus signs. More recently the highest proportion of sodium chloride has been found to occur simultaneously with the peak of circulating oestrogen, therefore obvious ferning patterns would be expected to occur predominantly during the follicular phase and in particular, during oestrus (Luño et al. 2012; Wei et al. 2013). This explains the absence of mucus ferning during the luteal phase and pregnancy as the high progesterone levels are negatively correlated with oestrogen levels (Wearing 1959; Yilma & Sobiraj 2012).

Previous studies have described up to three patterns for classifying crystallisation. The three types are classified as either A, B or C-type ferning in cows (Abusineina 1962). A pattern with long, filamentous central stems and obvious venation over 95% of the slide (Type A) occurs 24-40 hours before ovulation and are often found in pliable, transparent mucus samples that are easy to extract. Type B patterns are characterised by short stems with irregular or absent

venation over 50% of the slide and are evident in the proestrus period, 3-5 days prior to ovulation and 4-5 days afterwards. Type C patterns occur throughout the remainder of the oestrous cycle with irregular ferns or no pattern forming (Abusineina 1962). These three classifications enable differentiation between proestrus and oestrus. The Type A pattern is present prior to ovulation and therefore, this technique could be capable of identifying the optimum time for insemination (Glencorse et al. 2017). A similar classification protocol detected a dense, filamentous fern pattern present in cervical mucus samples collected from sows 36-48 hours prior to ovulation (Luño et al. 2012). Several studies identified a crystallisation pattern that is present at the onset of behavioural oestrus, with the signal occurring 24-48 hours prior to ovulation (Abusineina 1962; Glencorse et al. 2017; Luño et al. 2012). This suggests that the gradual increase in oestrogen during early oestrus is causing the composition changes (Edström 2009). Despite these results, a clear and simple crystallisation classification protocol has not been developed or tested in commercial settings as the technique is still somewhat subjective, with different observers providing varied or opposing assessments of a sample (Aboul-Ela et al. 1983). This limitation could be overcome by testing alternative classification guidelines and implementing on-farm training. This alternative oestrus detection tool could be implemented on-farm with minimal training and equipment requirements, and in doing so, provide an objective, quantifiable and universally identifiable set of guidelines.

1.5.2 Electrical resistance (ER)

Electrical resistance (ER) is an electrical measurement that has been used to detect the changes in mucus composition that occur throughout the different stages of the oestrous cycle (Cassar et al. 2011). Mucosal tissues conduct an electrical charge which can be measured by introducing a current into the epithelial lining (Zink & Diehl 1984). This charge is a result of the presence of an increased concentration of sodium and chloride ions which provides a greater intensity of ions through which electrical currents can be conducted (Aboul-Ela et al. 1983, Ko et al. 1989). As the vaginal epithelium undergoes hydration, an increase in the electrolyte content in mucus causes an elevation in the electrical conductivity of the tissue 48 hours prior to behavioural oestrus which is a medium that can be detected by certain ER technologies (Yilma & Sobiraj 2012; Zink & Diehl 1984).

Many different devices have been developed specifically to measure the electrical potential which is measured in ohms and produced by mucosal tissue in response to a current that is applied to the mucosal surface as it travels between multiple electrodes (Ko et al. 1989; Řezáč 2008). The observed fluctuations during the oestrous cycle show a peak ER during the luteal phase in conditions of minimum oestrogen concentration indicating that elevated progesterone severely reduces mucus production and conductivity (Aboul-Ela et al. 1983; Dusza et al. 1996; Yilma & Sobiraj 2012). A reduction in resistance values occurs during the follicular phase as oestrogen increases with ER remaining at a basal level for 2-3 days during proestrus and oestrus (Ko et al. 1989; Yamauchi et al. 2009). Most sows recorded basal resistance levels 1-2 days before obvious behavioural oestrus signs occurred (Hidalgo et al. 2015). The pre-ovulatory LH surge occurs 6.2±4.5 hours after a subsequent increase in ER (Yamauchi et al. 2009). While this indicates a potential marker for predicting the time of ovulation, the variability present between sows indicates that this may not be a precise method (Hidalgo et al. 2015). Further research needs to be undertaken to ensure that the precise point of ovulation can be detected using ER (Gupta & Purohit 2001). There are several factors that impact on the precision of ER probes.

1.5.2.1 Probe location and use

Electrical resistance values fluctuate at different regions within the female reproductive tract (Řezáč 2008). The resistance is higher when measured within 10 centimetres of the vulva and progressively decreases as readings are taken closer to the cervix (Aboul-Ela et al. 1983; Řezáč et al. 2003a; Řezáč et al. 2003b). Similarly, vestibular and vaginal resistance have contrasting changes during the oestrous cycle (Leidl & Stolla 1976). During proestrus, vaginal resistance decreases while vestibular resistance increases (Řezáč et al. 2003b; Zink & Diehl 1984). The reason for the contrasting changes is unknown. The practical application of this information is that consistency of probe placement is of the utmost importance to ensure results are valid (Cassar et al. 2011; Yamauchi et al. 2009). Different sized sows may have significant differences in the length of the reproductive tract which could introduce variation that prevents accurate detection of timing (Dybala et al. 2008). To overcome this issue, separate trials must be conducted to determine the effect of size on electrical resistance. In addition, previous

research has used ER to detect optimum insemination timing but there have been no studies using vestibular electrical resistance (Yamauchi et al. 2009).

The resistance of a tissue can only be detected if there is contact between the mucosal layer of the vaginal epithelium and the probe (Hidalgo et al. 2015). If contact between the epithelial surface and the probe is not maintained, conductivity will become varied and inconsistent (Řezáč et al. 2003a). In addition, the effectiveness of the probe in detecting resistance can be affected by an abundance of air trapped within the confined space of the vagina which can adjust the values to a higher level (Zink & Diehl 1984). It is crucial that there is consistent and full contact between the probe and the tissue (Řezáč et al. 2009).

1.5.2.2 Human error

Stock-people must be competent in using ER probes, particularly due to the importance of probe location and the contact point for obtaining accurate ER measurements (Cassar et al. 2011). Variation is also expected to occur between ER probes with differing specifications as well as in commercial settings where various operators use the same equipment (Řezáč et al. 2003a). These issues do not indicate that the method is imprecise for detecting oestrus, but it does highlight the need for training to ensure the correct use of equipment to measure ER accurately.

1.5.2.3 Disease

There is sow-to-sow variation evident in baseline vaginal ER recorded outside of an oestrus event. Vaginal ER was recorded over a range of 200-550 ohms in sows undergoing normal reproductive cyclicity (Glencorse 2013). Sows with low baseline vaginal ER values during the luteal phase where found to have significantly reduced farrowing rates, suggesting possible issues with hormone regulation or damage to the reproductive tract (Řezáč & Olic 1988). This information can be used to determine thresholds for culling sows that are not reproductively sound and thus increasing the efficiency of the entire herd (Bono et al. 2013). Research has indicated that diseases such as urinary tract infections can also shift the values for resistance higher (Zink & Diehl 1984). Continuously high VER without fluctuations can be attributed to ovarian cysts (Řezáč 2008). Changes in resistance only occurs when the maturing

follicles release a large amount of oestrogen and then rupture following the LH-surge (Yilma & Sobiraj 2012).

1.5.2.4 Parity

The parity of the sow also affects the VER reading. Sows with parities greater than two will have higher resistance readings than sows which have yielded a single litter, as older animals tend to have damage to the epithelial walls, meaning reduced permeability of circulating oestrogen into the cells (Kemp et al. 1998). In addition, older sows which are presumably larger in size will have longer reproductive tracts which can impede connection between the tissue and probe at the required location (Gama & Johnson 1993). As a result, the basal resistance indicative of oestrus is less pronounced in older sows and therefore may interfere with accurate identification of ovulation timing (Řezáč et al. 2009).

1.5.2.5 Insemination protocol

Despite the comparable farrowing rates obtained using VER probes for detecting the optimum time for insemination, there has been limited implementation of this technology in commercial settings, primarily due to the lack of improvement in farrowing rates (Glencorse 2013; Řezáč et al. 2009; Yamauchi et al. 2009). This is particularly important as multiple inseminations are still required due to limited precision when using twice daily observations for oestrus detection (Řezáč et al. 2009; Zink & Diehl 1984). In addition, the collection of data from VER probes requires collation of daily readings to ensure that the decreased VER value is identified (Dusza et al. 1996). This technology has not previously been capable of consistently improving conception rates above existing techniques and therefore has not been adopted by the wider industry. However, these technologies warrant further investigation as they have the potential to quantify previously subjective and inconsistent techniques, hence overcoming variation that impedes reproductive efficiencies (Hidalgo et al. 2015).

Previous studies performed artificial inseminations with chilled spermatozoa 12 and 24 hours after a detectable increase in VER leading to a farrowing rate of 85% (Ko et al. 1989), 80% (Yamauchi et al. 2009), 70-75% (Zink & Diehl 1984) and a non-return rate of 70% (Řezáč & Olic 1988). These results are comparable to farrowing rates obtained after behavioural

detection of oestrus which resulted in farrowing rates of 75% (Yamauchi et al. 2009). This correlation supports the use of VER probes as an accurate oestrus detection tool (Glencorse 2013; Ko et al. 1989).

1.5.3 Body Temperature

Body temperature, a measurement used for identifying health status, can be used for monitoring physiological changes to normal bodily functions (Bressers et al. 1994). Temperature monitoring in sows has allowed detection of farrowing, disease and oestrus (Luño et al. 2013).

The fluctuations in body temperature are different based on the location on the body (Schmidt et al. 2013). Radio-telemetric devices implanted under the skin are highly accurate and obtain continuous data. They have been used to detect an increase in mean ear temperature 6-12 hours before oestrus in cattle (Bressers et al. 1994). However, subdermal measurement of temperature in cattle is more variable than rectal and tympanic areas as superficial tissues are influenced by ambient environmental conditions (Hahn et al. 1990). An alternative non-invasive thermometer that requires minimal training is an infrared thermometer (Osawa et al. 2003; Sykes et al. 2012; Talukder et al. 2014). This technology uses a handheld pointer device which obtains accurate temperatures by utilising infrared waves to measure below the surface of the skin (Soerensen & Pedersen 2015). This process is simple, quick, repeatable and hygienic and also minimises sow distress by preventing the need for physical contact (Scolari et al. 2011). While infrared technology is the most accessible method for temperature monitoring, the use of more advanced thermometers can reduce the noise from ambient temperature and improve the accuracy of this oestrus detection method (Luño et al. 2013).

Thermal technology can be used to measure temperature across an entire surface instead of being limited to a single point when using an infrared thermometer (Schmidt et al. 2013). This process involves photographing the body location and using software to calculate the average temperature (Talukder et al. 2015). This provides a greater ability to accurately detect the true temperature of the location without environmental effects (Simoes et al. 2014). It can be applied to areas such as the vulva which becomes swollen and red and is expected to undergo

an associated temperature change during oestrus (Simoes 2012). This technology would enable the detection of oestrus specific temperature changes that could be used as a marker for insemination timing (Sykes et al. 2012). It also has the potential to be used for identification of other physiological events such as parturition and disease (Green et al. 2008; Luño et al. 2013). Early detection of these factors can improve production efficiency if stock-people can be made aware of individuals that require directed treatment or assistance (Bressers et al. 1994). To identify the effectiveness of body temperature monitoring in a commercial setting, the results obtained using both simple, handheld infrared thermometer devices or a more advanced automated thermal system should be compared.

Automated detection of changes to baseline body temperature can be detected using video-based thermography (Talukder et al. 2014). The main benefit of using video-detected temperature is that it can collect continuous data at much smaller time intervals than a handheld device (Kashiha et al. 2013a). In addition, handheld devices require a consistent technique, significant animal contact and are more laborious (Scolari et al. 2011). Automated thermometer recording can identify individuals that have shown a specified temperature that requires action or intervention (Saint-Dizier & Chastant-Maillard 2012). As a result, there are minimal labour requirements as stock-people are notified to enable action (Fisher et al. 2008). The anatomical positioning of the vulva may impede automated technologies as faeces, which is often present on or near the vulva, can affect the accurate recording of body temperature (Soede et al. 1997b).

Ear temperature has been measured at the base of the ear in sows (Bressers et al. 1994). Similarly, body temperature is often measured in the rectum as this location correlates closely with core body temperature (Green et al. 2008). Another potential location for observing body temperature is the vulva. During oestrus there is an increase in blood flow to the area resulting in hyperaemia and as blood infiltrates the surround capillaries, an elevated vulva temperature is observed (Simoes 2012; Sykes et al. 2012). These fluctuations in vulva temperature have been observed in cows (Aoki et al. 2005; Fujomoto et al. 1988) and sows (Simoes 2012). Peak vulva temperature was detected between 12-36 hours prior to ovulation in sows using a digital infrared thermometer (Scolari et al. 2011). In addition, vulva temperature was found to decrease significantly by 1.14°C from baseline 12 hours prior to ovulation (Luño et al. 2013). These changes have been attributed to the elevated concentration of oestrogen which causes

swelling and associated temperature changes to the reproductive tract (Czaja & Butera 1986). While this is useful information, the reason for the decrease in temperature prior to ovulation is unclear. The use of vulva temperature changes has been observed during oestrus, but current research has not investigated fluctuations in other body locations. Using temperature fluctuations in more obvious body locations as a signal for oestrus could be beneficial.

1.5.4 Quantification of behaviour

As the average herd size increases, individual contact between animals and stock-people is reduced, leading to several animal welfare concerns, an issue that is of particular importance with growing trends in group housing of sows (Cornou & Lundbye-Christensen 2008). Direct observation of animals is required to identify stress, pain, disease, discomfort, feed intake, reproductive capacity and growth and by limiting observation time per animal, changes in these parameters can go unnoticed (Ruiz-Garcia et al. 2009). By detecting and reporting on behaviour discrepancies using automated technologies, compromised growth can be detected early and long-term repercussions prevented (Schön et al. 2004). The large herd size of commercial pig farms has encouraged the use of precision technology to improve labour allocation, maintain animal welfare standards and production efficiency (Bressers et al. 1994; Kashiha et al. 2013b). Several technologies have been developed for automated, precision monitoring of domesticated and production animals. There are a range of uses for precision technologies in relation to reproductive performance (Chanvallon et al. 2014).

1.5.4.1 Infrared technology

Infrared photocells were one of the first methods used to quantify parturition behaviour and allow for automatic detection of parturition (Jones 1966). This technology allowed for differentiation between standing and lying behaviour based on opposing light beam patterns that are detected by a range of infrared photocells, paired with mirrors and linked with a remote computer system (Erez & Hartsock 1989; Jones 1966). Posture changes were found to be more frequent during parturition than during non-parturition times in sows housed within stalls (Hansen and Curtis 1981). A similar design was used to determine the peak number of posture changes which occurred 4-6 hours before parturition begins. Mean time spent standing per hour

was approximately 10 minutes/hour during late gestation followed by an increase to 20 minutes/hour in the 24-hour period prior to parturition (Barczewski 1987). This technology demonstrates that behaviour observations are an alternative to relying on observation of physical changes to mammary glands, the currently used method for determining the onset of parturition (Yun et al. 2013). This task is inaccurate and requires repeated observations by trained observers to detect the timing of oestrus accurately (Erez & Hartsock 1989). This system can predict parturition to within 12 hours by identifying high frequency posture changes over an extended period of time, hence reducing the requirement to repeatedly monitor individual animals during gestation (Erez & Hartsock 1989). These sensors can be used to predict farrowing in sows and in doing so, avoid piglet death and sow discomfort (Oliviero et al. 2008).

There are several other applications of precision technology that have been tested. Automatic image-based monitoring of water intake can allow observation of environmental conditions, disease, and feed intake (Kashiha et al. 2013b) and nest building behaviours have been detected by using infrared sensors (Jensen 1993). These applications have limited practicality for reproductive processes. However, one possible application is automatic classification of reproductive behaviours using acceleration technology which detects activity and motion continuously over a period of time (Cornou & Lundbye-Christensen 2008).

1.5.4.2 Time lapse video

Time-lapse video footage has been used to identify the frequency and duration of boar visitations by sows, whereby 95% of sows demonstrated standing heat, and signalled with sufficient time for a mating to be completed (Bressers et al. 1991). However, in this study of 50 animals, 42% of the sows had a false positive oestrus event detected on one or more occasion, which demonstrates low specificity (Bressers et al. 1991). This technology has been refined to allow more direct, quantifiable measures of boar visitation and oestrus length (Ostersen et al. 2010). Collar-based transponders were used with nearby receivers to detect and record the frequency and duration of visits to a boar. Peak visitation occurred 2-3 days after the first observed increase in the frequency of visits (Bure & Houwers 1989). While this is useful information for breeding management, it is usually only obtainable retrospectively.

Electronic sow feeder stations, time lapse video footage and transponder data are largely obtained retrospectively and therefore are incapable of real-time oestrus detection (Bono et al. 2013). In addition, these technologies rely on sows having access to the boar at all times (Cornou 2006). Full boar contact for 24 hours per day is absent from most commercial systems, particularly as it leads to habituation and a subsequent reduction in the behaviour expressed (Kemp et al. 2005).

1.5.4.3 Electronic oestrus detection station

Electronic identification of sows is necessary in welfare friendly production systems, particularly in group housing, outdoor housing and reduced confinement sow pens (Blair et al. 1994). One study used collar-based transponders linked with electronic oestrus detection (EED) stations. This system defined oestrus detection as an increase in the number of visits to an EED station located adjacent to a boar pen and found a correlation between conventionally detected behavioural oestrus and EED based boar visitation lengths ($r>0.05$, $P<0.05$) during consecutive oestrous cycles (Blair et al. 1994). Despite the correlation between boar visitation and conventional oestrus, the incentive for visitation to the EED station may be based on habituation to feeding systems that these sows were previously exposed to, indicating the need for further exploration (Paolucci et al. 2008). Using a form of electronic oestrus monitoring system can improve detection by 8% when compared to conventional behavioural detection, resulting in a reduction in the number of non-productive days per sow (Korthals 1999). Future studies should identify the relationship between hormonal fluctuations and boar visitation during oestrus. This would determine if there is a correlation that could be manipulated as a predictor of the correct time for insemination.

1.5.4.4 Radio-based monitoring systems

Radio-based electronic calving monitoring systems have been used to detect physiological states in cows. These systems are surgically attached to the vulva to detect altered radio waves as a calf exits the reproductive tract and sends an alert via mobile networks (Paolucci et al. 2008). This automatic detection of a change in the physical state of livestock results in high sensitivity detection of 100% and assists in real-time decision making and labour allocation (Marchesi et al. 2013). Generally, automated systems do not require interpretation and hence

allows less experienced stock-people to determine reproductive status while reducing the likelihood of false positive oestrus detection or missing an oestrus event completely (Wathes et al. 2008). Similar technologies could be developed to detect activities such as flank nosing and sow-sow mounting, which is often linked with early stages of oestrus (Schön et al. 2004).

1.5.4.5 Pedometers

One application of motion-based technology is the use of pedometers. Commercial products, such as IceTag3D™, provide a step count and accumulative motion index based on internal algorithms of acceleration magnitude (Nielsen et al. 2010). Sows often display heightened activity levels and increased levels of interaction with other sows during oestrus (Hemsworth & Tilbrook 2007). This technology would be particularly helpful for individual sows that demonstrate silent oestrus when other specific behavioural changes are not displayed during oestrus (Brown et al. 2002; Henriksen et al. 2005; Moreira et al. 2001). This information could be exploited by using pedometers to detect increased activity levels and hence signal the onset of oestrus (Fisher et al. 2008). This is an application that has been successful for a reduced activity level in cattle experiencing lameness (Nalon et al. 2013; Pastel et al. 2006). However, the mode of attachment is important as pedometer technologies did not enable classification of the step-taking motion observed in cattle with a pedometer attached to a collar-based electronic feeding system (Tani et al. 2013). This is because mutually exclusive leg and body movement cannot be differentiated when the sensor is placed on the head (Nielsen et al. 2010). Placement of sensors on legs can enable differentiation between body and leg movements and hence identify the number of steps taken (Thompson et al. 2016). However, the attachment of sensors to sows is difficult in locations such as the ear, leg or face due to potential interference between normal behaviour and the motions the system is attempting to detect (He et al. 2008). In addition, retention rate of sensors is reduced in sows that are group housed or when insecurely attached (Cornou et al. 2006).

1.5.4.6 Accelerometers

Behaviours change to represent the physiological or reproductive status of an animal (Andrews et al. 2015). For example, nest-building behaviour is used to signal the onset of parturition (Jensen 1993); reduced activity levels indicate illness or disease that could be linked

with a variety of conditions such as lameness (de Mol et al. 1999); a lower feed intake can signal bacterial infections (Forbes 1995); and an increase in activity level signals the presence of sexual behaviour (Freson et al. 1998; Geers et al. 1995). Changes in behaviour are often the first visible sign to indicate issues with sickness (Andrews et al. 2015), stress (Papailiou et al. 2008), feeding patterns (Hartel et al. 2011), social and hierarchical changes (Cornou et al. 2011), parturition and birth (Marchesi et al. 2013) and oestrus (Cornou et al. 2006b). Improved efficiency in livestock production is possible by identifying and classifying these behaviours using large-scale data collection precision technologies (Alvarenga et al. 2016). In order for these technologies to be able to detect biological and physiological events, an understanding of the movement and behaviour associated with these events must be defined.

Accelerometers are small devices that can give quantifiable signal profiles for physical activity (Esliger & Trembley 2007). The hardware within an accelerometer converts movement-based oscillations into electrical signals (Tani et al. 2013). An acceleration force produces a voltage output that is amplified and converted into a digital value that is corrected for the effects of gravity (Andrews et al. 2015). This technology has been applied in several species to quantify the orientation of an object and the change in positioning over time that occurs as a result of movement. This has allowed a profile of several actions and motions, including human motion (Hendelman et al. 2000; Staudenmayer et al. 2009; Trost et al. 2000), feeding behaviour in dairy cows (McGowan et al. 2007), post-surgery behaviour in dogs (Brown et al. 2010), normal and feeding behaviour in sheep (Alvarenga et al. 2016) and illness in cats (Watanabe et al. 2005). Single-axis accelerometers have been used to successfully record and distinguish specific biting and chewing actions in dairy cattle and sheep (Dobos et al. 2016; Tani et al. 2013). The detection of rumination action allows for monitoring of typical feed consumption behaviour and identification of health issues that interfere with normal feeding activity. However, these accelerometers are often limited as they can only detect one plane of movement and therefore more detailed activities cannot be quantified.

Three-dimensional accelerometer technologies can detect posture and movement, at highly frequent data points (Nielsen et al. 2010). Tri-axial accelerometers use three planes of movement; vertical swing, medio-lateral and cranio-caudal movement (Cornou & Lundbye-Christensen 2008). Accelerometers attached to sows for a continuous length of time have been

used to form unique signal profiles of activity on three planes for several activities; walking, feeding, rooting and lying (Cornou & Lundbye-Christensen 2010). Walking was detected by a single profile showing forward acceleration, while lying sternally and laterally was identified by a reduction in the vertical acceleration and a single-sided horizontal biased acceleration respectively (Cornou & Lundbye-Christensen 2010). Despite the success of differentiation between these movements and postures, walking and rooting behaviours are often difficult to separate (Kashiha et al. 2014). Differentiation of these behaviours would be beneficial, with walking being indicative of correct motion and the absence of lameness while rooting indicates healthy feeding patterns (Cornou & Lundbye-Christensen 2008). To identify more complex motion events, a cumulative activity level can be used, instead of relying on a unique signal profile (Alvarenga et al. 2016).

Sensors can overcome the time-consuming, labour intensive nature of continuous behavioural observation, a task that is often not feasible in animal production, particularly in large herds (Tani et al. 2013). However, technology that is currently available does not automatically detect a signal change and alert the required personnel as it requires further engineering (Oliviero et al. 2008). To ensure that these behaviours can be identified and classified by accelerometers in sows, further studies are required to quantify the acceleration involved in each activity of interest and to enable development of automated systems for notifying stock-people. While accelerometers allow identification and quantification of behaviours which exploit the information that is readily available, the efficacy of accelerometers is affected by a range of factors.

1.5.4.6.1 Correct prediction

One aim of accelerometer technologies is to identify a signal that can be used to predict the associated behaviour (Martiskainen et al. 2009; Oliviero et al. 2008). However, the signal that is obtained is not always capable of predicting the presence of a behaviour with 100% certainty (Cornou 2006; Saint-Dizier & Chastant-Maillard 2012). The correlation between accelerometer signals and observed activity often yields high percentages of true positive predictions but this is usually accompanied by a portion of false positives and false negatives (Alvarenga et al. 2016). These false predictions are often a result of individual variation in

behaviour expression and could be caused by factors such as disease, weight, social interactions and differences in temperament (Alvarenga et al. 2016).

1.5.4.6.2 Weight

There are differences in signal profiles from accelerometers based on variation in the weight of individuals (Andrews et al. 2015; Preston et al. 2012). This is believed to occur because an individual with a larger mass can produce a greater force that increases the intensity of a movement (Dobos et al. 2014). This could be used as a definable variable if it is possible to estimate and predict a behaviour of interest for a sow based on a specific weight range.

1.5.4.6.3 Gait

Gait is the pattern of movements an animal is capable of forming to allow motion (Vázquez Diosdado et al. 2015). This range of movements is often highly variable in humans and monkeys due to their bipedal locomotion (Papailiou et al. 2008). This gait can introduce variation as simple movements such as walking and standing are often erratic, unbalanced and asynchronous and can be different based on the individual involved (Lee & Lee 2002). In contrast, movement in quadruped species is more consistent and usually based on simple, repetitive patterns with a full range of uninhibited movements (Andrews et al. 2015). When this information is considered, the accuracy of accelerometer-based behaviour prediction is higher in quadrupeds (Andrews et al. 2015).

1.5.4.6.4 Attachment site

Optimising attachment site of the sensor to the animal is important due to the need to ensure that data is collected continuously and effectively (Brown et al. 2012). Attachment of any electronic system needs to be secure to ensure continuous data collection and to prevent injury from consumption or contact with the transponders involved (Artmann 1999). Successful attachment in some species is not always straight forward and is substantially more difficult in social and wildlife species (Tani et al. 2013). Sensors attached to the horn, nose and forehead of cows using medical tape have been used to determine the location that can accurately detect rumination behaviours most accurately (Tani et al. 2013). The accelerometer attached to the

horn was most accurate, detecting 99% of observed activity (Tani et al. 2013). However, 40-61% of detected behaviours were false positives, with no difference between the three locations. This over-estimation of true events suggests that chewing behaviour may be falsely identified as rumination due to similarities in the signal profiles. Electronic ear tags have the potential to maintain the original purpose or simple detection of a specific behaviour (Artmann 1999; Hansen et al. 2007). Specialised ear tags have been used on sows with minimal pain during application and no further injury following healing (Hernandez-Jover et al. 2008; Leslie et al. 2010). However, ear-based attachment can introduce variation in the movement detected by the accelerometer across multiple species (Vu et al. 2017). Individual pigs have varied ear size and conformation based on differences in breed which could introduce discrepancies in movement intensity and how the actions are completed (Watanabe et al. 2007).

Collar-based accelerometers have been used successfully for classification and prediction of normal behaviour in domestic cats (Andrews et al. 2015), sheep (Alvarenga et al. 2016; Dobos et al. 2014), dairy cattle (Rutten et al. 2013) and individually housed sows (Cornou & Lundbye-Christensen 2010). However, the use of collars in group-housed sow systems is difficult, as shown by a study where 44 occasions of removal by the pigs occurred within a group of 40 gilts (Blair et al. 1994). The proposed reason for these difficulties was based on differences in attachment and adjustment strategies used by individual personnel as well as the social interactions and temperament of the sows (Edwards et al. 1984; Escalante et al. 2013).

1.6 Thesis aims

Commercial AI protocols using liquid-stored spermatozoa require accurate oestrus detection to ensure high conception rates occur within a herd. While oestrus detection is successfully conducted throughout the pork industry, reproductive efficiency is variable due to inability to accurately determine when ovulation occurs, unskilled labour and limited and subjective oestrus detection protocols. An additional issue as the global demand for food continues to grow is the concurrent increase in average herd size as agricultural systems are forced to become intensively focused with larger animal to stock-person ratios. This suggests that protocols for detecting the correct time for insemination must become simpler, faster and more accurate to fit within existing commercial operations. Novel procedures for detecting oestrus with a high level of accuracy and precision are essential for optimising productivity.

One possible approach to this involves identifying and quantifying physiological markers associated with ovulation instead of focusing on the broader oestrus period. The aim of this thesis is to quantify the physiological and behavioural changes that occur during oestrus and to determine any correlation between the markers and the optimum timing of insemination. Each chapter will investigate a different mechanism associated with oestrus. The studies will determine if the markers are directly correlated with ovulation itself or with the immediate time-points prior to ovulation. These links would determine if the marker could be used alongside or instead of existing oestrus detection protocols as an objective signal for insemination timing. There are several tools and techniques available that can quantify visible or detectable changes to conductance in reproductive tissue, cervical mucus, body temperature and physical activity. These changes will be observed during the broad oestrus period as well as the time adjacent to ovulation.

Chapter 2 will investigate and measure the success of vaginal electrical resistance of reproductive tract tissue using commercially available probes. Chapter 3 will assess changes to cervical mucus composition including pH, viscosity, biochemical analyses and visible microscopic pattern classifications throughout oestrus. This chapter will assess a unique set of visual and descriptive guidelines for classifying crystallisation patterns found in cervical mucus smear samples. In addition, the effectiveness of these guidelines for broad application will be

investigated through a survey to determine if previous scientific training is required for successful application of these guidelines. Chapter 4 will investigate body temperature of the forehead, ear and vulva using two thermometers; an infrared gun and a thermal camera to determine the most reliable technology for measuring oestrus-associated changes in body temperature. Chapter 5 will quantify physical behaviour by using tri-axial accelerometers to identify unique signal profiles for detection of behavioural events and to measure cumulative activity levels during the oestrus period. These quantified physical changes will be observed alongside faecal hormone profiles to determine if there are any markers that, when used to detect the optimum time for insemination, provide improved or comparable detectability of the reproductive state of the sow.

Finally, Chapter 6 will assess the combined predictive power of the individual physiological markers with the aim of producing a new oestrus detection protocol that encompasses multiple techniques. The markers involved in this chapter will be identified based on an observed correlation with ovulation from the previous chapters. This approach will improve precision when deciding on insemination timing, while also reducing stock-person labour requirement.

The outcomes of this thesis will substantially improve oestrus detection protocols by determining methods that are capable of assisting or replacing current oestrus detection methods in order to improve conception and farrowing rates in commercial systems. These techniques and tools have the potential to reduce the number of semen doses required per sow, minimise reliance on boar presence and improve consistency in stock-person training. The physiological markers examined in this study have the potential to enable more precise identification of the optimum insemination timing.

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Chapter 2

Electrical resistance of the vagina and vestibule

Chapter 2
Using vaginal and vestibular electrical resistance as an alternative marker for optimum timing of artificial insemination with liquid-stored and frozen-thawed spermatozoa in sows

2.1 Introduction

Artificial insemination (AI) in sows is a process that requires multiple doses of semen, delivered daily due to the uncertainty in accurately detecting ovulation (Knox 2016; Martinez et al. 2002). Conventional AI in sows involves a labour-intensive monitoring program for detecting oestrus by visual observation of behavioural indicators such as flank nosing, snout contact, urogenital sniffing, mounting of other sows and the presence of standing heat in response to back pressure (Behan & Watson 2005). Physical changes such as vulva redness and swelling or discharge from the vulva are also used in oestrus detection programs (Rath et al. 2008). These observations are time-consuming, often variable between animals and require substantial training due to their subjective nature, leading to difficulty in identifying these changes (Roca et al. 2006). More accurate methods focused on detecting ovulation instead of the general oestrus period would allow stock-people to achieve more consistent insemination outcomes. (Rozeboom et al. 2004). Additionally, an objective tool could provide an additional benefit of reducing the number of semen doses if the exact moment of ovulation can be detected (Bracken et al. 2003; Garcia et al. 2007).

Inseminations with frozen-thawed spermatozoa require more precise detection of the specific timing of ovulation rather than just identifying the oestrus period (Vazquez et al. 2005; Waberski et al. 1994). Frozen-thawed spermatozoa has a post-thaw lifespan of approximately 16-20 h less than liquid-stored spermatozoa (Rath et al. 2008). Liquid-stored spermatozoa can survive in the female reproductive tract for up to 24 h while frozen-thawed spermatozoa have a limited lifespan of approximately 4-8 h (Abad et al. 2007; Luño et al. 2015; Vazquez et al.

2005). The reduced lifespan causes a reduction in the motility and affects DNA integrity leading to substantial effects on farrowing rate and litter size (Fraser et al. 2011; Lewis & Aitken 2005). These issues lead to a shortened window of opportunity for semen deposition and therefore an increased requirement for oestrus detection labour when implementing frozen-thawed artificial insemination (FTAI) programs (Didion et al. 2013; Roca et al. 2011). One potential method for overcoming these issues is through monitoring of electrical resistance levels in the vagina and vestibule (Luño et al. 2013; Yamauchi et al. 2009; Yilma & Sobiraj 2012).

Electrical resistance (ER) is a value that quantifies the opposing force that impedes the flow of electrical current through an object (McCaughey & Patterson 1981). The resistance of an object is determined by the size and material each object is made from (Gabriel et al. 1996). A positive or negative charge is produced by each object and this can be measured by introducing an electrical current to the surface of the object (Feldmann et al. 1978). This concept can be applied to the measurement of mucosa epithelium conductivity (Ko et al. 1989; Smith et al. 1989). Electrodes implanted in the reproductive tract of cows determined a reduction in the electrical resistance during the oestrus period (Feldmann et al. 1978). This is because an increase in the vascularity of tissues in the reproductive tract leads to fluctuations in the electrical potential of the surface (Talukder et al. 2018). Vaginal ER has been monitored throughout oestrus and has been shown to be correlated with increasing concentrations of oestrogen in the Okinawan native Agu pig (Yamauchi et al. 2009). A profile of vaginal ER in sows identified a drop to the minimum level during the early stages of the follicular phase, followed by a sharp increase to peak levels in the early luteal phase (Ko et al. 1989; Yamauchi et al. 2009). These vaginal ER changes are well correlated with fluctuating oestrogen and LH concentrations (Dusza et al. 1996). Comparable conception rates of 85% have been obtained using conventional oestrus detection and vaginal ER-predicted oestrus detection, when double inseminations of liquid-stored spermatozoa were performed (Glencorse 2013; Zink and Diehl 1984). However, the use of vaginal ER for predicting frozen-thawed insemination timing has not been successful, with conception rates dropping to 40% (Glencorse 2013).

Greater FTAI fertility may be possible by identifying biological markers associated with ovulation instead of using conventional behavioural observation to predict insemination

timing (Roca et al. 2011; Waberski et al. 1994). Farrowing rates of 80% and litter sizes of 10 can be obtained when frozen-thawed spermatozoa are inseminated within 4-8 h of ovulation by experienced technicians (Eriksson et al. 2002; Spencer et al. 2010). However, these fertility results are only achievable when observations occur at 4-hourly intervals (Ringwelski et al. 2013). Frozen-thawed AI is not commonly used in the pig industry due to the requirement for frequent oestrus observation and the relatively low fertility obtainable compared with liquid-stored semen (Roca et al. 2006).

This study will determine if the electrical resistance readings in the vaginal and vestibular regions are associated with the behavioural observations that are typically used for oestrus detection and the hormonal changes associated with these changes. The aim of this study was to determine if ER in the vagina and vestibule can be used as a predictor for ovulation and to validate this procedure as an alternative oestrus detection method for commercial sow production.

2.2 Materials and Methods

Chemicals were sourced from Sigma unless otherwise stated.

2.2.1 Animal Management

The methods involved in this study were approved by the Animal Ethics Committee at The University of Sydney (2013/5942). Data was collected from Large White, Landrace and Duroc crossed sows (n=36) at The University of Sydney Mayfarm piggery in Cobbitty. The experiment included multiparous sows with a mean parity of 4.3 ± 1.7. Boars were housed in single pens, allocated ad libitum water and a low energy ration. Sows were batch housed in large deep litter pens in groups prior to parturition in farrowing crates with ad libitum water and a high energy lactation ration. Body condition score (BCS) was recorded upon entry to the farrowing house. They were moved from farrowing crates to batch housing in groups of 4-6 sows based on a pen size of approximately 14 m^2.

2.2.2 Experimental design

Sows were split into three treatment groups based on different proposed oestrus detection and insemination protocols: control/natural boar mating (n=14), liquid-stored AI (n=8) and frozen-thawed AI (n=7). The natural mating group underwent traditional behaviour-based detection of oestrus followed by joining with a breeding boar for a double insemination protocol at 0 and 24 h after the first recorded instance of behavioural oestrus. The liquid-stored and frozen-thawed groups had behavioural oestrus recorded but were inseminated according to vaginal ER-detected oestrus protocols as demonstrated in Yamauchi et al. (2009). A double dose protocol was used with inseminations conducted 0 and 24 h and 12 and 24 h after a detected increase in vaginal ER for liquid-stored and frozen-thawed treatments, respectively.

The four timings that were examined included the onset of behavioural oestrus, 24 h after the onset of behavioural oestrus, the estimated instance of ovulation as predicted by faecal hormone concentrations, and 24 h prior to predicted ovulation (Figure 2.1). In all treatment groups, an individual sow was deemed to be in oestrus if the standing heat response was observed following application of back pressure in the presence of a mature boar. The first instance of behavioural oestrus or the onset of oestrus was defined as 12 h before the first observation of standing heat in response to back pressure from the stockperson. The instance of predicted ovulation was determined by identifying the concentration of progesterone obtained in faecal collections. This time-point was defined as 30 h prior to the peak concentration of progesterone, which accounted for dispersal of hormones in the faeces and faecal transit through the digestive tract as demonstrated by Snoj et al. (1998).

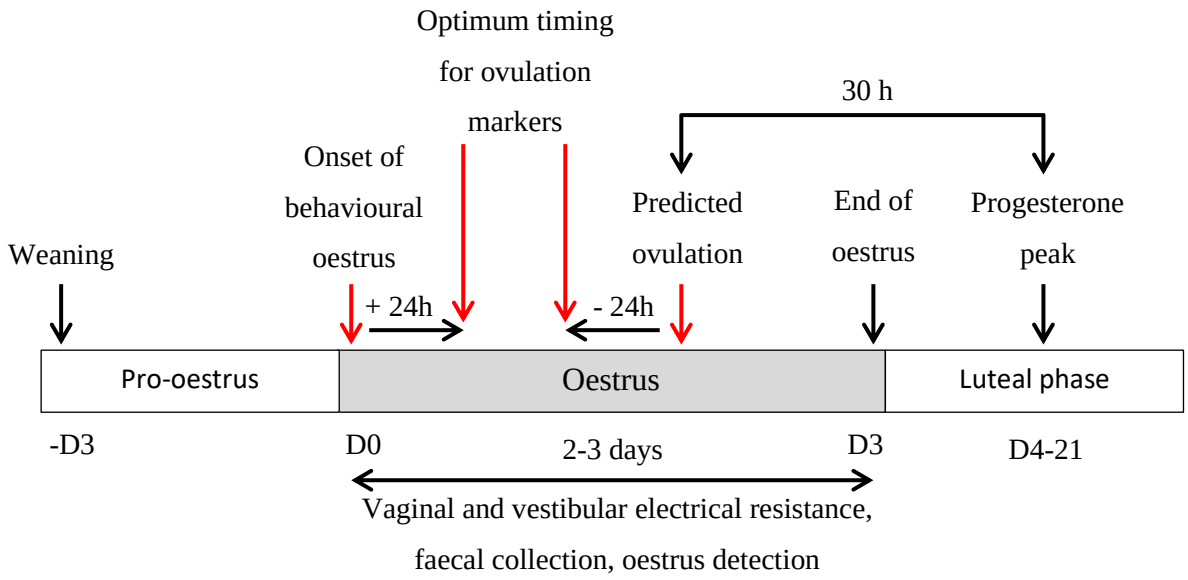

Figure 2.1 Schematic of the timings of examinations conducted during oestrus in sows to identify if electrical resistance is an effective physiological marker to determine optimum insemination timing. Day 0 is the first time-point in the oestrous cycle and is defined as the first instance of behavioural oestrus. The horizontal arrows lead to time-points that exist at an appropriate time relative to oestrus events and hence will enable identification of the physiological markers that could predict ovulation accurately.

2.2.3 Electrical Resistance

Electrical resistance of the reproductive tract was monitored twice each day (07:00/15:00) using a Draminski resistance probe (Draminski-Electronics, Olsztyn, Poland). The probe was disinfected in an iodine solution and dried before and after use in each sow. The sow's vulva was cleaned, and the probe inserted into the vagina on an upward angle of 20-35° until resistance from the cervix was encountered. The probe was operated as per manufacturer's instructions (Cassar 2011). The electrical resistance value for the epithelium of the vagina and the posterior epithelium of the vestibule was recorded.

2.2.4 Oestrus detection and insemination protocols

Oestrus detection was recorded from three days after weaning until two days after the last instance of behavioural oestrus. Sows were observed for 30 mins twice a day (07:00/15:00) to identify oestrus behaviours including standing heat, flank nosing, snout contact, sow-to-sow mounting and urogenital sniffing. The protocol for detection of oestrus for the control group involved movement of sows to a stall near a mature boar for five mins. Individual sows were deemed to be in oestrus if the standing heat response was observed following application of back-pressure in the presence of a mature boar. The liquid-stored and frozen-thawed treatment groups underwent vaginal ER-detected oestrus. This procedure involved daily recording and monitoring of ER from the vagina and vestibule, with oestrus defined as the first increase in ER following basal level readings.

Control sows were joined with a sexually mature boar in a designated mating area at 0 and 24 h after standing heat was first detected, with assistance provided to boars during copulation. Both liquid-stored and frozen-thawed treatment sows were moved to a stall alongside a mature boar and inseminated according to the treatment with Melrose style catheters depositing semen into the cervix (Minitube, Tiefebach, Germany). Semen was collected and either frozen-thawed, or liquid-stored prior to being used for insemination. The semen collected from these boars was used as separate doses with no semen pooling.

2.2.4.1 Liquid-Stored Spermatozoa

Semen was collected from the boars using the gloved hand method into a filtered collection bag held in a pre-warmed 37°C thermos flask. Only ejaculates displaying motility greater than or equal to 80% were used. Semen was extended with Androstar Plus (Mintube, Germany) to make AI doses of 3.5 x 10^9 sperm in 80ml and cooled to 15°C over 2 h. The spermatozoa were maintained at this temperature for 3 and 4 days until use.

2.2.4.2 Frozen-Thawed Spermatozoa

The sperm-rich fraction of semen was collected from a boar using the gloved hand method into a filtered collection bag held in a pre-warmed 37°C thermos flask (King & Macpherson 1973). Only ejaculates with motility of 80% or greater were used. The semen was

extended (1:3 semen:Androstar Plus) and cooled to 15°C over 2 h. The extended semen was centrifuged at 800 x g for 20 min at 15°C and the resultant pellet was resuspended in cooling extender (80% v/v of 11% w/v lactose solution with 20% egg yolk) to give a concentration of 1.5 x 10^9 spermatozoa/ml. The suspension was cooled to 5°C over 1.5 h, when a freezing extender (89.5% cooling extender supplemented with 1.5% (v/v) Equex STM (IMV, L'Aigle, France) and 9% glycerol) was added to give a final concentration of 1 x 10^9 spermatozoa/ml. The spermatozoa were frozen in 0.5mL straws using a programmable freezer with the following settings: 5°C to -6°C at -3°C per min held at -6°C for 1 min and cooled from -6°C to -140°C at -50°C per min. Samples were immediately plunged into liquid nitrogen (LN_2) for storage until use. The straws were thawed by agitating in a 37°C water bath for 40-60 secs until liquefied and warm to touch. The 15-18 straws that were thawed yielded a single dose of 3.5 x10^9 motile spermatozoa for insemination and AndrostarPlus was slowly added to make a final volume per dose of 80ml. Frozen-thawed samples were only used if motility was greater than or equal to 40%.

2.2.5 Faecal progesterone assay

Faecal samples were collected from each sow and stored at -80°C until further processing. Each sample was dried at 65°C overnight or until dried throughout. The samples were crushed into a fine dust and large contaminants such as straw were removed. Samples were extracted using ethanol or ethyl acetate at a rate of 1 mL/0.1 gram (Sigma-Aldrich 24102-1L-R, >99.8%). All samples were mixed overnight and centrifuged at 4,200 x g for 15 mins. A 500 µL volume of supernatant was transferred to a clean tube for evaporation in a SpeedVac vacuum oven. The dried extracted samples were frozen and stored at -20°C in a desiccator prior to use in a DetectX® Progesterone ELISA Kit (Arbor assays, Michigan, USA).

Extracted samples were mixed with 100 µL of ethanol followed by 400 µL of assay buffer (1:5 dilute AB concentrate: deionised water). The samples were vortexed three times and allowed to sit at room temperature for 5 mins and diluted with 1-5mL of ethanol and assay buffer. The reconstituted diluted samples were run in duplicate in a 96-well assay plate. A 25 µL volume of progesterone conjugate (DetectX® progesterone-peroxidase conjugate in stabilising solution, Arbor Assays) and 25 µL volume of progesterone antibody (DetectX® mouse progesterone specific monoclonal antibody, Arbor Assays) were added to each well and

mixed thoroughly. A plate shaker was used to mix the contents of the wells for 2 h. The plate was aspirated five times with wash buffer (1:20 dilution of wash buffer concentrate to deionised water) before adding 100 µL of TMB substrate (3,3',5,5' - tetramethylbenzidine, Arbor Assays) to each well. The samples were incubated for a further 30 mins before adding 50 µL of stop solution. The optical density of each sample was recorded using a plate reader (4PLC software) at a wavelength of 450 nm. The progesterone concentration was calculated based on a standard curve using 4PLC software.

2.2.6 Fertility Results

Sows were assessed for return to oestrus 21-25 days post-insemination. Transcutaneous ultrasound (SonoSite Vet 180 Plus) was performed 35 days after insemination and conception rates were recorded. The farrowing rate, litter size, piglets born alive (BA) and stillborn piglets (SB) were recorded post-parturition.

2.2.7 Statistical Analysis

Statistical analyses of the data were completed using the R statistical software package (i386 v3.4.2, R Core Team (2017)). The non-return rates and pregnancy rates were assessed using a Generalised Linear Mixed Model. This model indicates the probability of return to oestrus based on the effects of insemination type (natural mating, liquid-stored or frozen-thawed). A REML Linear Mixed Model was used to analyse the correlation between vaginal ER and timing within the oestrous cycle. Effect of probe location was assessed using correlation analysis in the form of Pearson's tests. A level of $P < 0.05$ indicated a statistically significant result for all tests.

2.3 Results

Of the 36 sows included in this study, 29 demonstrated behavioural oestrus and were inseminated according to the treatment allocation. Reproductive data of the cycling sows used in the study are shown in Table 2.1. The sows that did not undergo an oestrus event and therefore did not experience an insemination were removed from the experiment.

Table 2.1 Reproductive data of the cycling sows used to monitor electrical resistance during oestrus.

Sample sow data	Mean ± S.E.M	Range
Sample size	29	
Parity	2.41 ± 0.27	1-7
Body condition score (BCS)	3.12 ± 0.07	2.5-4
Wean-oestrus interval (days)	4.6 ± 0.42	2-7
Length of behavioural oestrus (hrs)	58.3 ± 0.48	49-68
Estimated ovulation based on behavioural oestrus (hrs)	37.0 ± 0.51	32-42

Electrical resistance underwent changes through the oestrous cycle (Table 2.2). A cyclic change was observed in both vaginal and vestibular electrical resistance with elevated levels detected in proestrus followed by a gradual decline in early oestrus and sharp rise 24 h prior to predicted ovulation ($P=0.023$). Both resistance measurements gradually decreased from the point of predicted ovulation until a plateau in the early luteal phase (Figure 2.2). Vaginal electrical resistance was positively correlated with vestibular electrical resistance ($P<0.001$; $r=0.655$). However, the variation in the vestibular measurements was greater than the vaginal measurements.

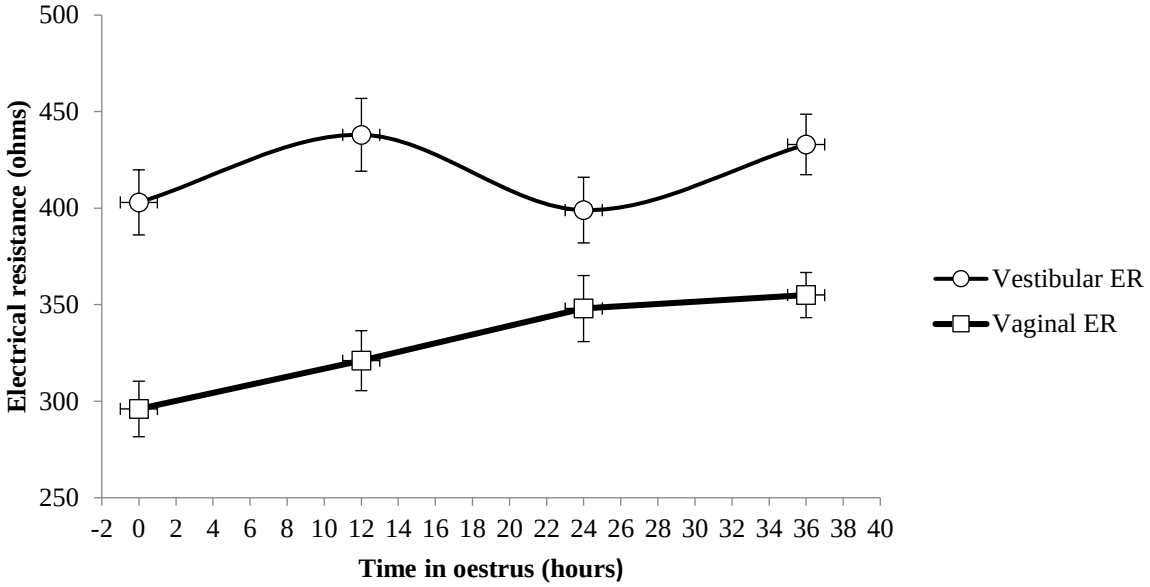

Figure 2.2 Mean (± S.E.M) electrical resistance recorded at two locations within the reproductive tract (vestibule and vagina) during the oestrus cycle. Time points are relative to the onset of behavioural oestrus detected by the presence of standing heat in the presence of a boar. The last data point coincides with predicted ovulation, which was defined as 30 h prior to the peak faecal progesterone concentration.

Table 2.2 Vaginal and vestibular electrical resistance of sows recorded at the onset of behavioural oestrus, 24 h after the onset of behavioural oestrus, the point of ovulation as predicted by faecal progesterone concentration and 24 h prior to predicted ovulation. The subscripts indicate a significance level of $P<0.05$ demonstrating statistical differences between time-points within one location.

Location	Onset of oestrus	24 hr pre predicted ovulation	24 hr post oestrus onset	Predicted ovulation
Vagina	296 ± 14.38^{a}	321 ± 15.52^{b}	348 ± 17.10^{c}	355.28 ± 11.70^{c}
Vestibule	403 ± 16.85^{a}	438 ± 18.86^{b}	399 ± 17.02^{a}	433 ± 15.67^{b}

The length of oestrus was consistent amongst the three treatment groups with no significant differences identified. All sows that were deemed to have conceived resulted in a successful pregnancy (Table 2.3). The conception rates and farrowing rates for the liquid-stored and frozen-thawed inseminations (both performed at timings determined by vaginal ER) were significantly lower than those for the control sows (mated according to commercial oestrus detection protocols). The ER predicted inseminations resulted in farrowing rates of 72.73% which was significantly lower than the control sows ($P=0.034$). Also, the number of piglets born alive per litter was less in the liquid-stored and frozen-thawed groups compared with the control group ($P<0.05$). Similar conception rates, farrowing rates and numbers of piglets born alive per litter were obtained for the liquid-stored and frozen-thawed inseminations ($P>0.05$). The number of stillborn piglets per litter did not differ among the groups ($P>0.05$).

Table 2.3 Oestrus, conception and farrowing rates obtained from control sows inseminated naturally using conventional oestrus detection, and sows inseminated with liquid-stored and frozen-thawed semen using ER to determine the timing of insemination. The superscripts indicate a statistical difference of P<0.05 between insemination types within each column.

Insemination type	Sample size	Oestrus length (h)	Conception rate (%)	Farrowing rate (%)	Born alive	Stillborn
Control	14	52.5 ± 2.2	90.91%[a]	90.91%[a]	12.1±1.6[a]	0.30
Liquid-stored	8	54.5 ± 2.1	72.73%[b]	72.73%[b]	9.5±1.6[b]	0.13
Frozen-thawed	7	57.5 ± 3.6	66.67%[b]	66.67%[b]	7.7±1.1[b]	0.17

2.4 Discussion

This study found clear correlations between vaginal and vestibular electrical resistance readings and the timing of ovulation, as predicted by faecal hormone analysis, suggesting that ER-detected oestrus is a promising ovulation detection tool for predicting the optimum AI timing. This is possible as elevated secretions of cervical mucus are associated with increased oestrogen production in the reproductive tract prior to ovulation which contributes to the elevated conductance of mucosal tissues (Hidalgo et al. 2015). These results support previous studies which indicated that changes in the readings from ER probes could be used to predict optimum insemination time and reiterate that AI should be conducted at the first instance of rising vaginal ER after the basal level followed by a second insemination 24 h later (Cassar et al. 2011; Dusza et al. 1996; Luño et al. 2013; Yamauchi et al. 2009).

The study is the first to observe a correlation between vaginal and vestibular ER and the peri-ovulatory period and as such this physiological change could be used for predicting AI timing. The lowest vaginal ER was identified at the onset of behavioural oestrus while the lowest vestibular ER of was recorded 24 h after the onset of oestrus. These trends indicate that both measurements could be used to predict the optimum time for insemination, with AI recommended 8-12 h after the first detected change in vaginal ER while change in vestibular readings signal the need for immediate insemination. Variations between the measurements

taken in the vagina and vestibule may be due to the composition of the cervical mucus present within these locations (Aboul-Ela et al. 1983). The components within mucus vary in relation to oestrogen concentration and as such the secretion is rarely consistent (Dusza et al. 1996; Luño et al. 2013). Mucus is produced and excreted from within the cervix with a homogenous consistency related to the concentration of oestrogen acting upon mucosal cells in the cervix (Haynes 1971; Lai et al. 2009). As oestrogen secretion increases gradually and fluctuates intermittently throughout oestrus, cervical mucus is produced in varied densities (Luño et al. 2012; Theodosiadou & Tsiligianni 2015). This produces small aliquots with varied compositions, but due to the disparate viscosities of these sub-samples, mixing and equal dispersion of the molecular components may be difficult, resulting in heterogeneous mucus (Lai et al. 2009; Pluta et al. 2012; Zaaijer et al. 1993). Low density fluids have a smaller mass that requires less energy to allow movement and vice versa (Freedman & Ibaraki 2002). As mucus moves caudally through the reproductive tract, dispersal of each sub-section of mucus is uneven, leading to the production of greater heterogeneity, particularly in the vestibule which is the most distal from the cervix (Bansil & Turner 2006; Lai et al. 2009). In addition, the cervix has a smaller lumen diameter than the vestibule which would reduce air interference (Hannallah et al. 1996). As a result, correct contact between the tissue and probe is essential for accurate measurements as air pockets prevent conductance of the electrical current (Řezáč et al. 2009). This indicates that the location and quality of contact with the ER probe is essential if using these tools in a commercial setting. Training of stock-people in ER probe use is essential for ensuring that accurate ER measures are obtained.

The current study observed a significant difference between both liquid-stored and frozen-thawed vaginal ER-detected insemination treatments and conventional behaviour-based oestrus detection. This experiment contradicted previous studies where inseminations involving liquid-stored spermatozoa led to conception rates of 80-90% (Glencorse 2013). Reduced conception rates with liquid-stored inseminations may be due to the wean-to-oestrus interval (WEI). Sows with a shorter WEI undergo an earlier onset of reduced vaginal ER readings that are concurrently followed by a sharp increase in readings, which is the signal for insemination to occur (Řezáč et al. 2009). As this change is the signal for conducting inseminations within ER-detected oestrus monitoring, there may be an issue with this method

due to the WEI variation introduced. Alternatively, the differing results obtained between the studies could be due to a boar effect or a small sample size for the ER-detected treatments.

Typical commercial insemination protocols using behaviour-based oestrus detection to determine the optimum timing for insemination result in conception rates of 85-100% and 40-90% for liquid-stored and frozen-thawed AI respectively (Abad et al. 2007; Didion et al. 2013; Soede et al. 1995; Waberski et al. 1994). Any replacement oestrus detection method should be capable of obtaining comparable or improved results in order to warrant adjustments to existing farm procedures. The current study observed a reduction in conception rates for liquid-stored AI but maintained acceptable rates for the frozen-thawed treatment group when compared to conventional AI outcomes. Using ER to predict when liquid-stored AI should be conducted caused a change in the timing of AI relative to oestrus onset and ovulation which may have led to lower conception rates for these groups. The oestrus length of sows in the liquid-stored treatment were shorter than those of the frozen-thawed treatment. A reduction in oestrus length is associated with ovulation at earlier stages of oestrus which could have contributed to the lower than average conception rates in the liquid-stored group (Belstra et al. 2004). Using ER to identify the optimum AI timing may be difficult in these sows as a reduced oestrus length would impact on the timing of ER changes, causing fluctuations to occur on a faster schedule and making detection difficult. When focusing on the frozen-thawed group, conception rates were comparable with those obtained commercially. However, as the expected lifespan of frozen-thawed samples is 4-8 h post-thaw, an ER oestrus detection protocol could lead to an absence of viable spermatozoa within the reproductive tract at the time of ovulation (Spencer et al. 2010). The current study performed frozen-thawed AI at 12- and 24 h intervals after a detected increase in vaginal ER, leading to a conception rate of 67%. This represents an improvement in conception and farrowing rates for frozen-thawed semen when compared with the rates reported in a previous study using vaginal ER to inform insemination timings (Glencorse 2013). The findings of previous studies have suggested that the limited lifespan of frozen-thawed spermatozoa reduces the conception success of inseminations to 20-50% when conducted 12 and 36 h after the first detected increase in vaginal ER (Yamauchi et al. 2009; Glencorse 2013). This refinement of the procedure could be implemented commercially within the typical workday of 8 h to enable the benefits of frozen-thawed AI to be achievable.

The measurement of vaginal ER often results in varied outcomes due to the inability to observe the location and proximity of the probe relative to the cervix (Řezáč et al. 2003). In addition, individual variation between technicians occurs based on their previous experience, developed skill and competency and can be problematic if training has not been conducted correctly (Řezáč et al. 2009). To overcome this variation in conductance measurements, ER can instead be recorded in the vestibule. Vestibular ER can be recorded more consistently as it enables the probe to be inserted into the reproductive tract the same distance in all animals, ensuring that measurements are comparable across all sows (Řezáč et al. 2003). However, parity and sow size could introduce variation within the vestibule (Řezáč et al. 2009). The process of parturition can cause damage to the reproductive tract, particularly in multiparous sows, which leads to inconsistent surface-probe contact and increased ER variation. Unequal lumen shapes and varied mucus quantities between sows can cause differences between the proximal and distal vestibular ER values recorded. This signifies that the probe must be inserted to a consistent depth in the vestibule during all observations (Luño et al. 2013; Řezáč et al. 2003). The volume of the vestibule should be investigated further to determine whether this contributes to the observed variation in vestibular resistance.

These results show that a minimum of two resistance measurements are required each day to accurately detect a change in conductance. Collection of this objective information is a short process with a labour requirement of 10-20 seconds per sow (Cassar et al. 2011). There is a substantial reduction in the labour required to complete this task when compared to traditional behaviour assessments, which allocate 10-15 mins of visual inspection for each sow to ensure accurate detection of oestrus events (Belstra et al. 2004; Kemp et al. 1998). The use of vaginal ER to detect oestrus removes the need to physically move sows to a designated mating area and visually observe both oestrus behaviours and interactions with boars (Langendijk et al. 2000). Additionally, this technique can be conducted without a boar present as the measurement encapsulates the physiological changes that are driven by hormonal changes throughout the oestrous cycle instead of momentary behavioural fluctuations that are stimulated in the sow in anticipation of copulation (De Rensis & Kirkwood 2016; McBride et al. 2019). These factors highlight the effectiveness of ER-detected oestrus because the limited

labour availability and restrictive timeframes in commercial situations impede precision in oestrus detection.

2.5 Conclusion

In conclusion, vaginal ER was found to be a more consistent marker for informing insemination timing than vestibular ER. The lowest vaginal resistance coincided with the onset of behavioural oestrus. Insemination at the first detected increase in vaginal ER over basal levels resulted in conception and farrowing rates that were similar for liquid-stored and frozen-thawed spermatozoa. However, these rates were significantly lower than those obtained using conventional oestrus detection and natural mating. Compared with previous ER studies, changing the timing of AI to 12 and 24 h after a detected increase in vaginal ER is proposed to improve the success of insemination using frozen-thawed spermatozoa. Given the short fertilizing lifespan of frozen-thawed spermatozoa, it is recommended that future studies implement double inseminations at 12 and 20 h after a vaginal ER increase.

The lowest vestibular resistance was detected 24 h after the onset of behavioural oestrus. Vestibular resistance could also be used as a marker for insemination timing, as the first increase over basal levels occurred approximately 12 h prior to ovulation. Identification of this time-point would allow a single insemination to be effectively scheduled such that the survival of spermatozoa for fertilisation is ensured. However, in a commercial setting, this physiological marker will not allow sufficient time for stock-people to conduct repeated daily inseminations prior to ovulation.

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Chapter 3

Cervical mucus composition: pH, viscosity, crystallisation and biochemistry

Chapter 3

Using cervical mucus characteristics to predict the optimum timing for artificial insemination

3.1 Introduction

Correct timing of artificial insemination (AI) is crucial to ensure sow breeding programs are efficient and profitable (Roca et al. 2011). Identifying the optimum timing of AI relies on efficient oestrus detection methods and insemination protocols differ according to the type of semen and the number of doses used to achieve conception (Alm et al. 2006; Knox 2016; Steverink et al. 1999; Waberski et al. 1994).

Liquid-stored spermatozoa can survive in the female reproductive tract for up to 24 hours (Kemp & Soede 1997; Nissen et al. 1997). Current AI protocols involve a minimum of two semen doses for each sow during an oestrus event following the first observed oestrus-based behaviour change (Cassar et al. 2005; Roca et al. 2006; Vazquez et al. 2008). Previous studies into the use of single doses showed reduced conception rates and litter size when compared with conventional multiple dose breeding programs (Garcia et al. 2007; Kraeling & Webel 2015; Roca et al. 2003). This is because oestrus detection protocols are incapable of determining the exact timing of ovulation (Soede et al. 1994). A single semen dose delivered at the first observed change in sexual behaviour will not be viable if the oocytes are ovulated 2-3 days later (Bracken et al. 2003). The number of doses of semen administered to each sow is a crucial factor that determines conception rate and litter size and relies on knowledge of the exact timing of ovulation (Frangež et al. 2005). The increased cost and labour associated with multiple insemination doses is inefficient and needs to be optimised (Lamberson & Safranski 2000). Identification of the specific timing of ovulation will enable a reduction in the number of semen doses that are necessary to ensure conception occurs without causing a reduction in the farrowing rate and litter size (Cassar et al. 2005). This concept has been examined from an alternate perspective by inseminating specifically designed capsules that enable the preservation and slow release of viable spermatozoa using a single insemination (Vigo et al.

2009). While this study enabled comparable fertility outcomes in the subsequent farrowing, these programs are not currently financially accessible to commercial producers, hence the need for enhanced oestrus detection protocols.

Identifying the ideal timing for insemination is also essential for optimising the use of frozen-thawed spermatozoa (Waberski et al. 1994). Frozen-thawed artificial insemination (FTAI) is not common in the pig industry due to the relatively low fertility obtained compared with liquid-stored semen when behavioural oestrus detection is used (Roca et al. 2006). Farrowing rates of 80% and litter sizes of 10 can be obtained when frozen-thawed spermatozoa are inseminated within 4-8 hours of ovulation by experienced technicians (Eriksson et al. 2002; Spencer et al. 2010, Waberski et al. 1994). However, there is a shortened window of opportunity for semen deposition and therefore an increased requirement for oestrus detection labour when implementing FTAI programs (Didion et al. 2013; Luño et al. 2015). Additionally, these fertility outcomes are only obtainable when observations occur at 4-hourly intervals and this is not practical in a commercial setting (Roca et al. 2006).

Identifying the optimum window for AI becomes a more difficult task as conventional procedures rely on detecting oestrus through observation of behavioural changes which indicate sexual receptivity (Belstra et al. 2004). The most commonly used behavioural observation used in the pig industry is a display of standing heat in response to applied back pressure (Weitze et al. 1994). This test is often conducted in the presence of a boar and it is most accurate when observed in combination with other behavioural indicators such as flank nosing, snout contact, urogenital sniffing and mounting of other sows (Soede et al. 2011). These observations are time-consuming, labour-intensive, require substantial training and experience and often provide inconsistent results between individual observers due to the subjective nature of analysing behaviour (Lamberson & Safranski 2000; Roca et al. 2006). Consequently, an alternative marker that provides more quantifiable and objective signals for insemination would be beneficial (Glencorse 2013). Improved fertility may be achieved by identifying biological markers associated with ovulation instead of using sexual receptivity to predict insemination timing (Roca et al. 2011; Waberski et al. 1994).

One possible marker that is associated with the oestrus period is cervical mucus (Betteridge & Raeside 1962). Cervical mucus undergoes oscillating patterns of change during oestrus which are associated with elevated oestrogen levels (Lavaud & Trouillas 2012). The physical and biochemical structure of the fluid is highly responsive to changes in the concentration of oestradiol (Luño et al. 2012). Previous studies have identified that cervical mucus undergoes gross composition alterations to the texture, caused by an increase in the density, viscosity and volume during oestrus (Betteridge & Raseide 1962; Haynes 1971; Lee et al. 2013; Zaaijer et al. 1993). The mucus is often described as sticky or tacky, a change that occurs because of increased mucin content leading to an increase in elasticity (Luño et al. 2012). These mucins have been found to assist in preparation of the surface of the uterus for attachment of the fertilised embryo (Carson et al. 1998). Furthermore, the cervical mucus is altered due to a larger proportion of dissolved substances including sodium chloride and phosphates, as well as the concentration of mucins and pH within the secretion (Bansil et al. 1995; Correa 2001; Luño et al. 2012; Platt 1966). These dissolved substances are arranged in a specific design within the mucus, which can be visualised under a microscope when the samples are smeared and dried (Cortes et al. 2012; Ježková et al. 2008; Pardo-Carmona et al. 2010). These patterns, often called crystallisation or arborisation patterns, have an arrangement of filaments with a central stem leading to multiple, smaller sub-venations which look similar to a fern-like shape (Aboul-Ela et al. 1982; Abusineina 1962; Luño et al. 2012).

The reason for this adjustment to the structure is believed to cause a corresponding change to the purpose of the reproductive tract (Stevenson et al. 1981; Tsuma et al. 1996). The complex structures that form in the mucus of the cervix and vagina change to firstly provide conditions that are conducive for sperm transit to maximise chances of conception and then to prepare the tract for implantation (Ceric et al. 2005). These smears of cervical mucus form patterns that differ throughout the oestrus cycle (Abusineina 1962; Luño et al. 2012).

Several studies have identified that the pattern of the mucus smears is aligned with oestrogen secretion (Elstein 1982; Luño et al. 2012; Pardo-Carmona et al. 2010). The highest concentration of oestradiol has been correlated with elevated levels of cervical mucus crystallisation alongside the increased concentration of dissolved salts (Wearing 1959). One study classified all mucus smears collected during oestrus using three distinct pattern categories

and identified that peak crystallisation occurred 36-48h prior to ovulation (Luño et al. 2012). While this provides a marker of when to conduct inseminations using double doses of liquid-stored spermatozoa, it does not provide a precise indicator of ovulation for conducting single dose or frozen-thawed inseminations (Waberski et al. 1994). Further refinement of the mucus crystallisation categories by producing visual and descriptive guidelines with more pattern types would be beneficial for determining specific molecular changes that occur during oestrus and to allow the prediction of more precise insemination timing.

Implementing the visual and descriptive guidelines relies on a good understanding of microscopy produced images. University students, graduates and scientific professionals undergo training to acquire skills for the use of a microscope, a skill that will assist in comprehension of the images used in this study (DebBurman 2002). In contrast, stock-people and farm workers employed within the pork industry have limited interaction with microscopes due to a lack of need for this technology in animal husbandry (Eastwood et al. 2004). This leads to the assumption that agricultural employees would require microscopy training to enable understanding of images produced from this technology. This could be problematic as these farm workers are the target audience for training and implementation of this oestrus detection method (National Research Council 1988).

Despite the previously established links between oestrus and cervical mucus characteristics, further exploration of commercial application of these physiological changes as markers for the timing of insemination is necessary. Therefore, this study will investigate several physiological changes to cervical mucus. The aim of this study is to compare cervical mucus characteristics of viscosity, pH, crystallisation patterns and biochemical composition to faecal hormone concentrations associated with an oestrus event. This will determine any association between these physiological markers and the point of ovulation in an attempt to identify new tools for predicting the timing of insemination. To determine if the application of these tools is viable for commercial farms, a training and skill acquisition testing survey will be conducted to compare the ability of workers from white-collar and blue-collar industries to analyse and classify cervical mucus crystallisation images. These procedures could be an effective alternative method for monitoring oestrus and determining optimum insemination timing in sows on commercial farms.

3.2 Materials and Methods

3.2.1 Animal Management

The methods involved in this study were approved by the Animal Ethics Committee (Project number: 2013/5942) and the Human Ethics Committee (Project number: 2016/3838) at The University of Sydney. Data was collected from Large White x Landrace crossbred sows (n=43) located at The University of Sydney Mayfarm piggery. The average parity for sows recruited into the study was 2.6 ± 0.23. Sows were housed in individual farrowing crates during parturition and fed a high energy lactation diet. After weaning, sows were batch-housed in cement-floored pens grouped in batches of 4-6 in a pen size of approximately 14 m^2. They were provided with a low energy, dry-sow diet and ab libitum water.

3.2.2 Experimental design

Physiological markers, being cervical mucus characteristics, and behavioural changes were observed in sows throughout an oestrus event, from three days after weaning until two days after the last observed instance of behavioural oestrus. Four time-points were identified as vital stages during the oestrus event which were used for decision making (Figure 3.1). The time-points were chosen as they are crucial stages during the oestrus event when production staff are required to evaluate the status of individual animals and decide on adequate actions, being either to perform or abstain from an insemination. The timed markers used were the onset of behavioural oestrus, 24 hours after the onset of behavioural oestrus, the estimated instance of ovulation as predicted by faecal progesterone concentrations and 24 hours prior to predicted ovulation. All of these time-points would be valuable instances to determine the effectiveness of differing cervical mucus characteristics as an alternative oestrus detection tool.

3.2.3 Oestrus detection

Sows were observed for 30 minutes twice a day (07:00/15:00) and in this period the occurrence of standing heat was recorded. Behavioural oestrus was defined by the presence of a successful back pressure test, which required applying pressure to the sows back in order to illicit a physical standing response without any incidences of retreat. The first instance of

behavioural oestrus or the onset of oestrus was defined as 12 hours before the first observation of standing heat in response to back pressure from the stockperson. To identify the end of oestrus, 12 h was added to the end of the last observation of standing oestrus. The instance of predicted ovulation was determined by identifying the concentration of progesterone in faecal samples. The estimated time of ovulation was calculated as 66% of the length of behavioural oestrus (Snoj et al. 1998). This time-point was defined as the event 30 hours prior to the peak concentration of progesterone, which accounted for dispersal of hormones in the faeces and faecal transit through the digestive tract as demonstrated Snoj et al. (1998).

Alternative oestrus behaviours were observed twice daily (07:00/15:00) for 30-minute observation periods. The presence or absence of these behaviours was recorded and included flank nosing (repeated nuzzling and upward thrusting of the flank of another sow with the snout), snout contact (direct head-to-head contact between two sows accompanied by upward pushing), urogenital contact (repeated burrowing action of the snout of one sow near the vulva of another) and mounting behaviour (sow-to-sow contact where the ventral surface of one animal stimulates the dorsal surface of another). Data was collected for parity, body condition score, wean-oestrus interval, non-return rates and farrowing rate.

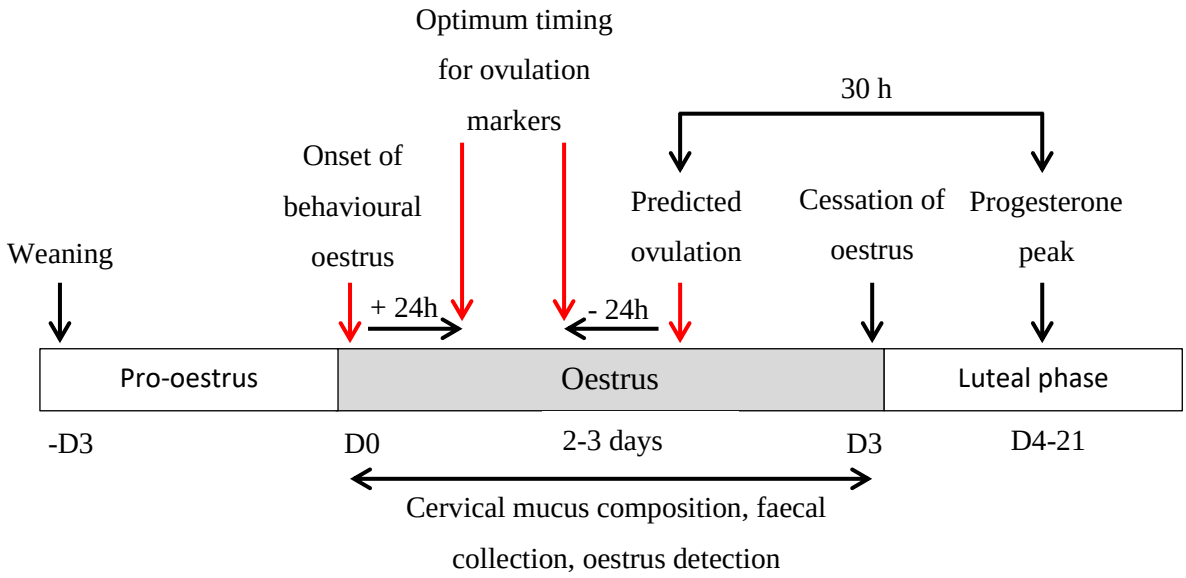

Figure 3.1 Schematic of the defined oestrus time points in sows used to identify if cervical mucus composition traits (pH, mucus viscosity, crystallisation patterns and biochemistry) are an effective physiological marker to determine optimum insemination timing. Day 0 is the first time-point in the oestrous cycle and is defined as the first instance of behavioural oestrus. The horizontal arrows lead to time-points that exist at an appropriate time relative to oestrus events and hence will enable identification of the physiological markers that could predict these events accurately.

3.2.4 Cervical mucus

Mucus was collected from the cervix of sows twice each day (07:00/15:00) using a 1.8 mL Rocket cervical mucus syringe (Rocket®, United Kingdom). The vulva was cleaned with water to remove faecal contaminants and dried prior to collection. The syringe was inserted into the vagina on an upward angle of 20-35° until resistance from the cervix was encountered. The end of the syringe was manoeuvred until it was possible to enter the cervix and mucus was drawn into the tube. The volume of cervical mucus, which ranged from 0.2-1.5mL, was split into four equal aliquots for further processing. One aliquot of the mucus was expelled onto a colour-based indicator pH strip (Sigma, USA) with the colour change immediately noted and recorded. The second aliquot was placed onto a microscope slide for a viscosity test which involved using a second slide to stretch the aliquot of mucus and measuring the length of the mucus thread at breakage. The third aliquot was stored at -80°Cin an Eppendorf tube. These

samples were centrifuged, and the supernatants were analysed in a sodium assay to determine the concentration in mmol/L. The final aliquot was placed onto a microscope slide and smeared evenly across the surface using a cover slip to form a thin film, as described below.

3.2.4.1 Crystallisation patterns

The thickness of the mucus on the slide was made consistent by pushing a cover slip across the surface to remove excess mucus. The mucus sample on the slide was allowed to air dry for one hour at room temperature before a cover slip was applied to the surface of the slide. The slides were stored in a refrigerator at 2°C for 2-3 weeks until they were examined under a light microscope (Olympus System Microscope BX40, Notting Hill, VIC, Australia). Each sample was imaged five times and each image was analysed for the predominant type of crystal pattern (Table 3.1) and the amount of space that the pattern covered in the field of view (Table 3.2) using Olympus Capture software (Windows 7 version). The predominant classification for crystallisation shape and coverage over the five images was recorded.

Table 3.1 Classification guidelines for identifying the predominant crystallisation pattern in a dried cervical mucus sample. Modified from Abusineina (1962) and Luño et al. (2015).

Score	Crystallisation shape description
1	No distinctive changes to the mucus structure
2	Short or long, straight or curved stems forming linear streaks with no venation
3	Small circular bubbles, individually or in aggregated clusters
4	Large irregular shapes, broken or random branching
5	Treelike branching, fern shapes with venation only
6	Distinct fern shapes with perpendicular branching, venation and sub-venation

Table 3.2 Classification guidelines for identifying the crystallisation coverage on a dried cervical mucus sample. Modified from Abusineina (1962).

Score	Crystallisation coverage description
1	Crystal patterns not present
2	Crystallisation pattern covers 0-20% of mucus sample on the slide
3	Crystallisation pattern covers 20-40% of mucus sample on the slide
4	Crystallisation pattern covers 40-60% of mucus sample on the slide
5	Crystallisation pattern covers 60-80% of mucus sample on the slide
6	Crystallisation pattern covers 80-100% of mucus sample on the slide

3.2.5 Faecal progesterone assay

Faecal samples were collected from each sow and stored at -80°C until processing. Each sample was dried at 65°C overnight or until dry throughout. The samples were crushed into a fine dust and large contaminants such as straw were removed. Samples were extracted using ethanol or ethyl acetate at a rate of 1 mL/0.1 gram (Sigma-Aldrich 24102-1L-R, >99.8%). All samples were mixed overnight and centrifuged at 800 x g for 20 min. A 500 µL volume of

supernatant was transferred to a clean tube for evaporation in a SpeedVac vacuum oven. The dried extracted samples were frozen and stored at -20 degrees Celsius in a desiccator until use.

Extracted samples were mixed with 100 µL of ethanol followed by 400 µL of assay buffer (1:5 dilute AB concentrate: deionised water). The samples were vortexed three times and allowed to sit at room temperature for 5 minutes. The reconstituted diluted 50 µL samples were run in duplicate. A 25 µL volume of progesterone conjugate (DetectX® progesterone-peroxidase conjugate in stabilising solution, Arbor Assays) and 25 µL volume of progesterone antibody (DetectX® mouse progesterone specific monoclonal antibody, Arbor Assays) were added to each well and mixed thoroughly. A plate shaker was used to mix the contents of the wells for 2 hours. The plate was aspirated five times with wash buffer (1:20 dilution of wash buffer concentrates to deionised water) before adding 100 µL of TMB substrate (3,3',5,5' -tetramethylbenzidine, Arbor Assays) to each well. The samples were incubated for a further 30 minutes before adding 50 µL of stop solution. The optical density of each sample was recorded using a plate reader (4PLC software, MyAssays Ltd) at a wavelength of 450 nm. The progesterone concentration was calculated based on a standard curve.

3.2.6 Visual and descriptive training and skill acquisition survey

Study data was collected and managed using REDCap electronic data capture tools hosted by the University of Sydney (Harris et al. 2009). The survey was distributed throughout the Australian pork industry in the Pork Bytes newsletter from the Department of Primary Industries and internally to staff and students at the University of Sydney. An email request to access the survey was sent to staff and students who were completing an undergraduate or postgraduate degree in the Sydney School of Veterinary Sciences during June-July 2017. Respondents were able to complete the survey via the link provided in the email.

The survey was developed to allow the communication of training guidelines to respondents and to assess their ability to follow the set of visual and descriptive guidelines for assessing images of cervical mucus crystallisation. The survey began with several screening questions to allow separation of responses based on the industry sector, education history and

previous experience with microscopy. In addition, a comparison of agricultural employees and science-based university students was used to identify the successful components of the two guideline types; descriptive or visual. Additional classifying data were collected including the current job role, length of employment and interaction with the pork industry. The survey was comprised of two sets of instructions; descriptive guidelines that used a single written sentence to define the pattern of interest and visual guidelines that requested the respondents to identify patterns similar to those presented in an image. Both the visual and descriptive guidelines had six categories to pick from in order to classify each image. A list of the questions and descriptive guidelines included in the survey are provided in Table 3.3 and the visual guidelines are provided in Figure 3.2.

Eight images of patterns that had been observed in a sample of sow cervical mucus were presented to respondents. The eight patterns were assessed by the author using both sets of guidelines and these answers were considered the correct, gold standard classification. These answers were compared to those obtained from respondents. The survey provided each image alongside the descriptive guidelines and then used the same images in a random order with the visual guidelines. Respondents were encouraged to choose a classification for each of the images.

3.2.7 Statistical analysis

Statistical analysis of the data was conducted using the R statistical software package (i386 v3.4.2, R Core Team (2017)). All data were expressed in the form of mean ± standard error of the mean (S.E.M) and a level of $P < 0.05$ indicated a statistically significant result for all tests. A REML Linear Mixed Model was used to analyse the cervical mucus traits relative to oestrus and ovulation. An ANOVA was used to identify associations between cervical mucus crystallisation and biochemical structure. Survey results were analysed using independent t-tests to compare means.

Table 3.3 Questions provided for respondents in an online RedCAP survey aimed at determining the effectiveness of visual and descriptive guidelines for classifying cervical mucus crystallisation patterns.

Number	Question
1	What is the highest level of education you have completed?
2	Which of the following describes your current occupation?
3	How long have you worked in your current role?
4	What is your current position/job title?
5	Do you currently or have you previously worked with the pork industry?
6-13	Please classify the image using the descriptive guidelines below. - No distinctive patterns - Short or long, linear shapes - Small, round, bubble shapes - Large, irregular shapes - Large, irregular, branching shapes - Large fern shapes with perpendicular branching
14-21	Please classify the image using the visual guidelines below.
22	How confident were you while using these guidelines?
23	How would you rate the descriptive guidelines?
24	How would you rate the visual guidelines?
25	Did you prefer the descriptive or visual guidelines?
26	Why did you prefer that guideline?
27	Do you think this type of evaluation would be useful to the pig industry?

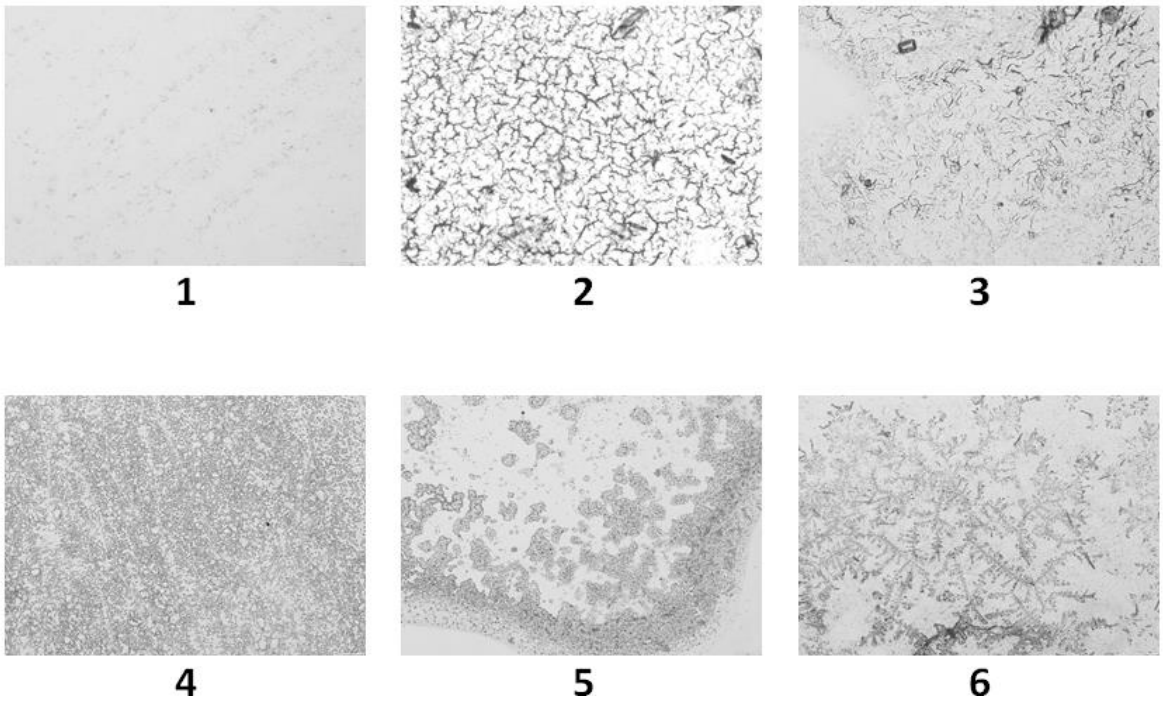

Figure 3.2 Visual guidelines provided for respondents in an online RedCAP survey aimed at determining the effectiveness of visual and descriptive guidelines for classifying cervical mucus crystallisation patterns with images representing the categories; **1** no pattern, **2** branching, **3** linear, **4** round, **5** irregular and **6** fern.

3.3 Results

3.3.1 Oestrus occurrence

Oestrus was observed in 43 sows over an 18-month period. The summary reproductive data for the animals included in this study are presented in Table 3.4.

Table 3.4 Reproductive data from sows included in the project measuring cervical mucus composition changes during oestrus (mean ± S.E.M).

Sample sow data	Mean ± SD	Range
Sample size	43	
Parity	2.60 ± 0.23	1-6
Body condition score (BCS)	3.05 ± 0.06	2-4
Wean-oestrus interval (WEI) (days)	5.2 ± 0.63	3-7
Length of behavioural oestrus (hrs)	52.2 ± 0.89	48-72
Estimated time of ovulation (hrs after the start of oestrus)*	34.5 ± 0.38	32-47

*Based on behavioural observations

3.3.2 Cervical mucus

3.3.2.1 Crystallisation of smears using light microscopy

The crystallisation pattern changed significantly over the duration of behavioural oestrus with the predominant pattern at the onset of behavioural oestrus being large, irregular shapes which changed to linear streaks 24 hours prior to predicted ovulation (P=0.013). In the periods prior to the onset of behavioural oestrus and after the final instance of behavioural oestrus there was no distinctive pattern category. The incidence of the remaining pattern types was not associated with any of the time points examined (P>0.05). The fern pattern was present in several sows after the onset of and prior to the conclusion of behavioural oestrus, but there were no significant correlations with this pattern at any of the time points examined. The two remaining patterns used in this classification, the branching and round categories, were found

sporadically and were not associated with any of the time points during the oestrus cycle (P>0.05).

3.3.2.2 pH and viscosity

Mucus pH values tended to decrease from 7.38 ± 0.78 at 24 hours prior to the time of predicted ovulation to 6.69 ± 0.58 at the time of predicted ovulation (P=0.091). There was no change in mucus viscosity from the onset of behavioural oestrus until the point of predicted ovulation.

3.3.2.3 Sodium concentration

Sodium concentrations of 114 ± 13.9 mmol/L were found in samples with irregular crystallisation patterns (Figure 3.3). This was significantly different to samples with linear patterns, which had a reduced sodium concentration of 48 ± 23.7 mmol/L.

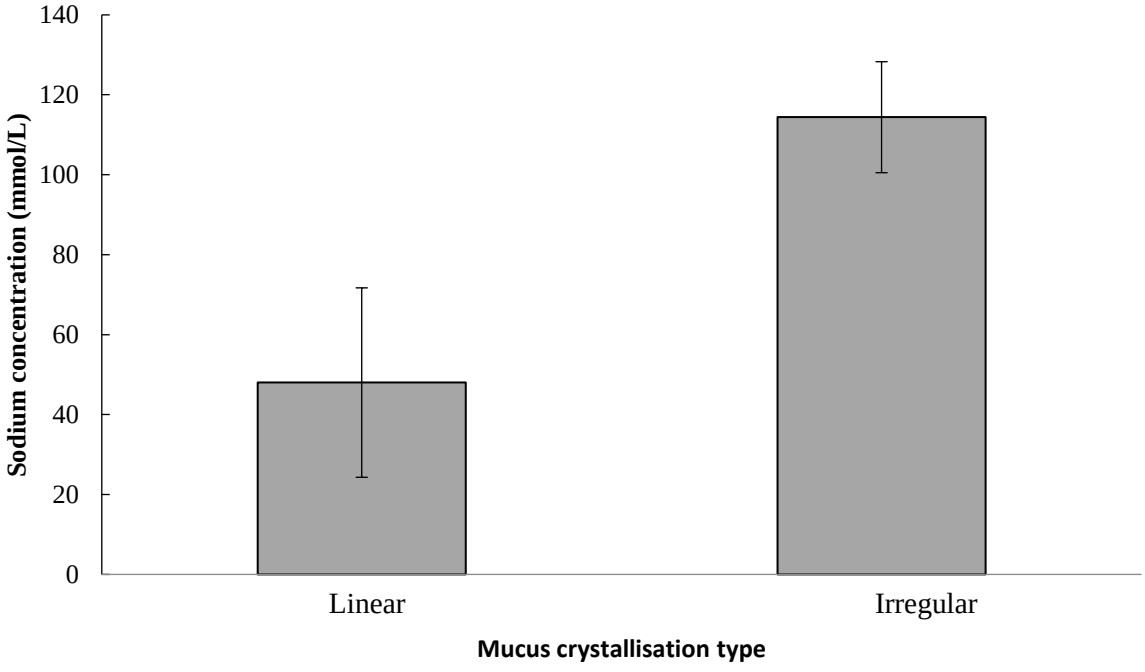

Figure 3.3 Sodium concentrations (mmol/L) measured in samples of cervical mucus that were classified as having linear or irregular crystallisation patterns.

3.3.3 Survey

A total of 70 responses were received from the voluntary survey. Of the 70 respondents, 57 completed the survey while the remaining 13 only provided answers to the screening questions and did not provide any answers to the classification questions for testing skill acquisition. The 13 incomplete surveys were removed from the analysis.

3.3.3.1 Education

Two respondents had completed a School Certificate or equivalent, while 12 respondents had completed a Higher School Certificate (HSC) or equivalent. The remaining respondents had completed additional education with five completing a Technical and Further Education (TAFE) course, 24 completing an undergraduate university degree and 14 completing a postgraduate qualification.

3.3.3.2 Industry

There were 18 respondents associated with the agricultural sector and 42 respondents from the scientific higher education sector. As the survey had directly targeted an audience that included agricultural and higher education sectors, the results obtained from these respondents were the focus of the further analysis. The remaining respondents were from other sectors.

3.3.3.3 Occupation

The results indicated that university students comprised a major portion of responses with 15 from undergraduate students and 13 who were studying for a PhD. There were 14 respondents employed at a university, six respondents were employed in agricultural production-based positions, while 12 were employed in a supervisory or managerial position. Of the survey respondents, 36% were currently working in the pork industry, 4% had previously worked in the pork industry and 60% were not working in the pork industry.

3.3.3.4 Comparison of descriptive and visual guidelines

The overall correct classification rate of all pattern categories using descriptive guidelines was 44% while the visual guidelines had a success rate of 62% (P=0.112). The visual

instructions produced significantly higher rates of correct classification for linear, fern, irregular and no pattern categories when compared with the descriptive guidelines (Figure 3.4). Branching and circle patterns had comparable rates of correct classification for both the visual and descriptive guidelines.

When using descriptive instructions, respondents had a high percentage of successfully classified samples for the round, irregular and no pattern categories while lower rates of correct classification occurred for linear and branching categories (Figure 3.5). However, the fern and irregular categories were detected with a moderate amount of success. Respondents determined linear, round, fern, irregular and no pattern categories with high rates of success when using visual instructions. The branching category was not successfully identified using the visual guidelines.

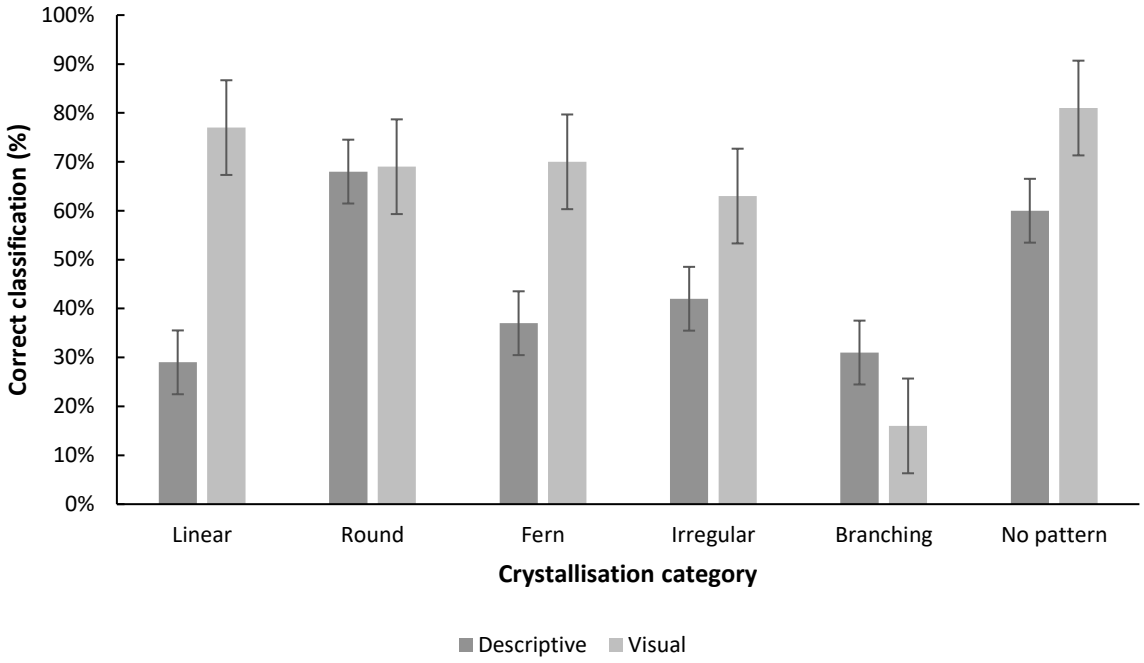

Figure 3.4 Percentage of correct classifications for the six categories of cervical mucus crystallisation by using either descriptive or visual guidelines.

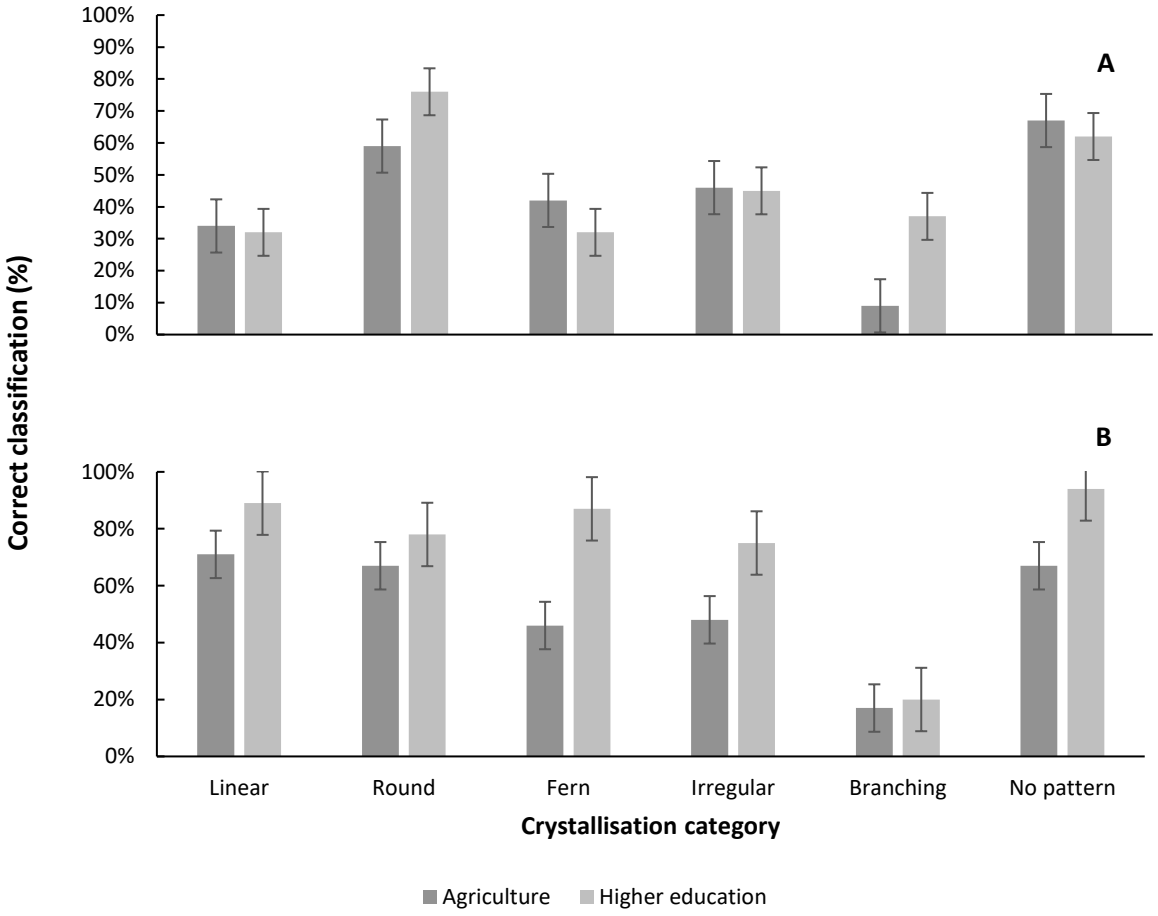

Figure 3.5 Percentage of correct classification for the six categories of cervical mucus crystallisation from different employment industries based on the provision of **A** Descriptive instructions and **B** Visual instructions.

3.3.3.5 Effect of employment industry

The lowest rates of correct classification for linear and branching patterns were observed from respondents whose highest qualification was a school certificate. The highest rate of correct classification of round and simple patterns was found in the group having TAFE and undergraduate qualifications.

When comparing workers in the agricultural sector to higher education industries, the percentage of correct descriptive classifications was similar for all patterns except for the round and branching categories. Alternatively, the visual guidelines had lower percentages of correct classification for the fern and irregular categories.

The two pattern categories that were predominantly associated with ovulation were linear and irregular shapes. The linear category was not identified differently based on employment industry for either the visual or descriptive instructed answers. However, there was a significant difference between the percentage of correct classification between the two instruction types. The visual guidelines resulted in significantly lower correct classification percentages from respondents in agricultural positions but there were no differences between employment sector when using descriptive guidelines.

3.3.3.6 Correct responses

The number of respondents that provided the correct classifications for both visual and descriptive assessments was higher for agricultural employees (Table 3.5).

Table 3.5 Percentage of respondents who provided both correct visual and descriptive answers for the six categories of cervical mucus crystallisation based on the industry of employment.

Employment industry	Linear	Round	Fern	Irregular	Branching	No pattern	Total
Agriculture	50%	82%	59%	61%	67%	92%	69%
Higher education	34%	69%	34%	46%	45%	64%	49%

3.3.3.7 Respondent opinions

Overall, 72% of respondents were somewhat confident when using either the visual or descriptive guidelines to classify images of crystallisation patterns (Figure 3.6). Both agricultural and higher education respondents demonstrated a preference for the visual instructions. The comments provided indicate that the descriptive guidelines were preferred due to certainty, objectivity and clarity while the visual guidelines were favoured due to simplicity and ease of use.

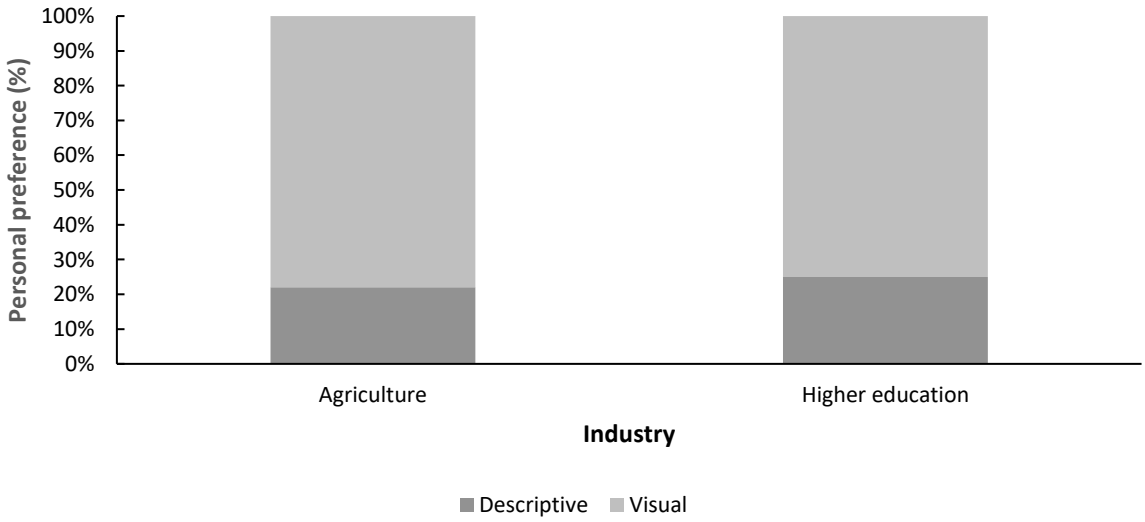

Figure 3.6 Personal preference of all respondents for either the descriptive or visual classification guidelines for the six categories of cervical mucus crystallisation based on different industries of employment.

3.4 Discussion

This study assessed a potential marker for insemination timing in the form of easily identifiable and distinctive mucus crystallisation patterns present 24 hours prior to ovulation. This marker could be easily recognised by non-scientifically trained survey participants and therefore would be a simple and effective method for detecting the optimum timing of insemination. The predominant crystallisation pattern underwent a shift with the average mucus pattern type 24 hours prior to ovulation moving from large irregular shapes at the onset of behavioural oestrus to short, linear streaks in the 24-hour period prior to ovulation. This study found that observation of crystallisation patterns is a valuable practice that could be implemented within the pork industry to enable producers to successfully detect the optimum timing for insemination. The current study identified six pattern types in cervical mucus collected from sows during oestrus, with three of these patterns from the onset of behavioural oestrus to the predicted time of ovulation. This change demonstrates that the micro-structure was altered, suggesting a reduction in the density of the mucus. This was also observed in a study by Luño et al. (2012) where peak crystallisation formation was observed 36-48 hours prior to ovulation followed by lower levels of crystal development approaching ovulation. This suggests that the mucus pattern becomes less structured to enable unimpeded movement of spermatozoa through fluid in the reproductive tract (Rutllant et al. 2005). This result corresponds with changes to the macro-structure of the mucus where the consistency becomes more aqueous in nature (Harding 1989; Lai et al. 2009).

Cervical mucus crystallisation has been examined in several different species (Cortes et al. 2012; Ježková et al. 2008; Luño et al. 2012; Pardo-Carmona et al. 2010). The results of these studies are difficult to compare as the categories used to define each pattern differ between studies and are often relatively subjective in nature (Noonan et al. 1975). However, the current observed change in the predominant pattern type from a dense to a sparse pattern, one day prior to ovulation has been previously identified in sows (Betteridge and Raeside 1962; Luño et al. 2012), cattle (Abusineina 1962) and dogs (Pardo-Carmona et al. 2010). One hypothesis for increased structural density in cervical mucus is the presence of circulating oestrogen, which has been found to trigger production of mucins (Rutllant 1997). Mucins are

large, glycoproteins which are organised into filamentous networks (Druart 2012; Odeblad 1997). As oestrogen concentration increases progressively through the oestrus period, the network of solid mucins separates and becomes sparse, leaving larger spaces for movement of aqueous fluids (Luño et al. 2012; Chretien et al. 1973). This corresponds with the micro-structure changes, indicating a potential relationship with the macro-structure that is helpful in detecting the correct time of insemination with more accuracy (Zaaijer et al. 1993).

While oestrogen has been proven to be related to the formation of cervical mucus crystallisation, the concentration of circulating oestrogen is rarely consistent (Odeblad 1997). This could show why the composition of mucus is not a homogenous substance (Luño et al. 2012). This has presented as a likely factor in the current study, with the methodology requiring detection of the predominant pattern in each sample. While this technique was an effective measure of the sample, it does not encompass the variation and heterogeneous nature of cervical mucus. Additionally, mucus becomes less homogenous in different sections of the reproductive tract (Menarguez et al. 2003). This indicates the importance of collecting samples consistently from the same location within the sows' tract (Řezáč et al. 2003). Any training for the use of this technique requires staff to be aware of the necessity for consistent collection site and method while also focusing on identifying the predominant pattern only.

The results presented here show correlations between peak circulating progesterone concentrations and the predominant cervical mucus crystallisation pattern. This differs from previous studies which have found that progesterone inhibits cervical mucus crystallisation, probably due to the lack of oestrogen, as oestrogen triggers an alteration in the composition of cervical mucus, forming the fluid that dries into crystal-like patterns (Luno et al. 2012; Wearing 1959). This discrepancy could be due to the differences in the classification guidelines used. The current study used six, clear, simple, shape-based categories for differentiating patterns that were observed in cervical mucus. Previous studies have used 2-3 categories, leading to issues with separation of physical appearance (Abusineina 1962). Similarly, Luno et al. (2012) used a three-pattern classification guideline and was able to identify a correlation between peak crystallisation at approximately 36-48 hours pre-ovulation.

The current study identified that samples classified as the irregular crystallisation type had significantly higher sodium concentrations than the linear pattern. Several dissolved substances are found within cervical mucus, including sodium chloride and bicarbonate salts (Gibbons & Glover 1959). The crystallisation procedure involves evaporation of the aqueous portion of the smeared sample by air-drying, and in the process the dissolved components remain, retaining remnants of the original structure (Abusineina 1962; Haynes 1971). This process identified an elemental change in mucus composition that can be used as a clear marker to separate two categories of crystallisation pattern, irregular and linear. The previously discussed correlation between linear patterns and circulating hormone profiles indicates that both crystallisation and sodium concentration could be used as physical markers to identify the optimum timing for insemination.

Implementation of cervical mucus sodium monitoring into an oestrus detection program would be simple. The use of biochemical markers in a pen-side test has significant benefits, including a reduced need for staff training due to the simple, objective and quantifiable guidelines. Methods could be devised to identify a peak or elevated level of sodium followed by a sharp decline. Inseminations that occur at the point of the decline in sodium concentration would ensure spermatozoa are deposited into the reproductive tract within 24 hours of ovulation. In order to use this information in commercial farms, a modified refractometer could be implemented to measure sodium content in a mucus sample (Calloway et al. 2002). Previous applications of this technology have enabled quantification of serum sodium concentration from blood samples from newborn babies with comparable results obtained from refractometer recordings and laboratory-based assays (O'Brien & Sherman 1993). Alternatively, near infrared spectroscopy (NIRS) could be used to measure the composition of mucus (Kleinebecker et al. 2013). Previous studies have developed specialised NIR procedures for various tasks including an oestrus detection procedure through measurement of milk and urine conformation and pregnancy testing through analysis of blood composition (Andueza et al. 2014; Agcanas et al. 2017). This technology could be applied in sow oestrus detection by using an NIR device as a rapid, pen-side test to detect mucus composition and the associated oestrus status.

Correct identification of patterns that are associated with ovulation is vital so this technique can be implemented for production staff on commercial farms with a high level of success. The survey revealed that the visual guidelines allowed 77% of the linear and irregular patterns to be correctly classified while only 29% of the descriptive analyses provided correct categorisation, thus demonstrating that visual guidelines are more effective and would be the preferred method for determining oestrus status in commercial production. These rates of correct classification were obtained without any training which suggests that this technique could yield greater results by providing basic training and support. Through the implementation of visually detected mucus crystallisation patterns, the need for multiple dose insemination programs could be reduced. This area should be a priority for future breeding management research.

The survey to test the effectiveness of the visual and descriptive instructions for skill acquisition when classifying cervical mucus crystallisation patterns indicated that visual guidelines were more effective than descriptive guidelines. Descriptive guidelines were limited in their success, with only 44% of samples classified correctly. The respondents identified both the round pattern and no pattern categories with a moderately high level of success while all other categories were identified with low success. Linear and branching categories were difficult to differentiate from each other. Similarly, there was difficulty in distinguishing between no pattern and round shapes. These outcomes may be due to limitations in the specificity of descriptors which were inadequate to accurately distinguish between categories that have similar visual characteristics. The descriptions in these categories need to be refined to ensure ease of understanding to prevent imprecise classifications. In contrast to the descriptive guidelines, the visual guidelines enabled respondents to differentiate patterns more successfully. Visual guidelines were used to correctly classify all categories except for branching with a high level of accuracy. In addition, the majority of respondents preferred these guidelines. This indicates that the visual guidelines are a better method for differentiating between mucus crystallisation patterns. This conclusion allows for better utilisation of this knowledge and increases the likelihood of improved rates of successful classification than previously assessed methods which focused only on written classification categories (Luño et al. 2012; Pardo-Carmona et al. 2010). The correct classification percentages ranged from 60-

70% for visual guidelines and 40-65% for descriptive guidelines, and so the precision of identification could be enhanced by utilising both instruction types for classification. To improve the detection of these pattern types in mucus smears for production workers, a combination of both visual and descriptive guidelines should be implemented together. This methodology accommodates for varied learning styles of individual respondents, as the combination of visual and descriptive guidelines will ensure that all individuals can comprehend the requirements for assessment of mucus crystallisation patterns.

Categorisation of crystallisation patterns using six groups is preferable to previous approaches in cattle and pigs (Abusineina 1962; Luño et al. 2012). The scope of previous research was limited as it focused primarily on defining the microscopic changes that occur during each stage of the oestrus event using 2-3 categories. This is the first study to attempt categorical classification of crystallisation patterns using six simple categories. Expanding the number of categories allows for greater differentiation of crystallisation patterns and therefore more targeted identification of the patterns that are associated with ovulation. This was demonstrated in the current study by the high rates of successful classification of pre-ovulatory mucus patterns.

Higher education respondents were better at classifying the crystallisation patterns than agricultural respondents, potentially due to exposure to microscopy training and greater development of skill level. This was particularly evident in the visual classification of denser and more complex fern and irregular patterns. High rates of accurate identification are necessary, particularly as the agricultural group will be required to implement these guidelines in commercial settings. Agricultural workers were able to correctly classify images for both visual and descriptive guidelines for all pattern categories except for branching with success rates of greater than 50% when using visual instructions. However, descriptive guidelines produced lower rates of successful classification in this employment group. While these results suggest that the success of the guidelines could be affected by previous microscope use, providing microscopy training and a quality control process to agricultural employees should allow for greater skill development that can improve the rate of consistent, correct crystallisation classification.

Classification of crystallisation patterns in cervical mucus is a simple method for detecting a physiological sign of oestrus that can enable accurate identification of the oestrus status of sows. This method can be implemented immediately on any commercial farm with ease as the mucus smear is simple to collect while also being possible at a low cost. However, there are several limitations of this method. The site of mucus collection must remain consistent to reduce inter-sample variation. The mucus must be collected from the cervix using a syringe capable of extracting the small volume of fluid that is produced. This requirement could be difficult to overcome unless sufficient guidance is provided to stock-people. Training should ensure a comprehensive understanding of the anatomy of the sow and the skills required to obtain a mucus sample.

Another constraint of this method is the labour requirement and time commitment of this method may be greater than traditional oestrus detection. Additionally, this method requires 1-2 mucus samples to be obtained each day from all sows. While the mucus test provides the benefit of more consistent oestrus monitoring, the additional labour may be problematic due to the shortage of skilled labour and temporary nature of employment in agricultural industries. However, once training programs have been conducted and sufficient time has been provided for stock-people to develop skills, the efficiency of mucus imaging may be more comparable with conventional oestrus detection methods. Alternatively, the length of this task could be reduced by optimising the methodology. The level of accurate classifications could be increased by automating the identification of crystal patterns using image recognition software. As the range of correct classification was different based on a variety of factors, this would also allow for removal of variation between individual assessors and create a completely consistent method. Machine learning has been used in the medical industry to allow diagnosis of acute lymphoblastic leukemia on microscopic images with 85-95% accuracy (Amin et al. 2015). This technique could be applied to crystallisation images in commercial production to reduce labour requirements for image assessment and improve efficiency by reducing the time required for classifying each sample.

3.5 Conclusions

Objective changes to cervical mucus were quantified during oestrus allowing for the identification of physiological markers that can determine the optimum insemination timing in sows. Mucus composition changed significantly throughout the oestrus period. The time point of 24 hours prior to ovulation, which indicates a suitable marker for conducting insemination, was correlated with a reduction in mucus pH, lower mucus sodium concentrations and the presence of short, linear streaks in dried samples. Additionally, the survey results indicated that agricultural production employees are capable of correctly classifying mucus samples with both descriptive and visual guidelines with a moderate level of success, respectively. The implementation of microscopy training and communication of visual and descriptive guidelines in commercial production would overcome the subjectivity of current behaviour-based oestrus detection protocols. By successfully identifying the point of ovulation using cervical mucus crystallisation categories, producers could overcome the need to conduct multiple dose insemination programs. These tools will be effective if implemented in an existing oestrus detection program to improve reproductive efficiencies, overcome skilled labour deficits and reduce the number of semen doses required for successful conception.

3.6 References

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Chapter 4

Body temperature

Chapter 4
Assessing thermal and infrared body temperature as alternative oestrus detection markers in sows

4.1 Introduction

Body temperature is usually maintained at a relatively stable and functional level known as the thermoneutral zone (Soerensen & Pedersen 2015). Alterations to the surface temperature of the skin can occur for a variety of reasons but usually relies on adjusted blood flow to the surrounding capillaries, causing a difference in the heat production (Sykes et al. 2012). Any temperature fluctuation away from the normal range can indicate an adjustment to the normal body functionality or a change in the physiological status of the animal (Bressers et al. 1994). These changes can signal parturition (Aoki et al. 2005; Fujomoto et al. 1988), injury or disease (Green et al. 2005) and oestrus (Luño et al 2013; Simões et al. 2014; Sykes et al. 2012). These temperature oscillations can be used as a physical marker for the physiological changes occurring in the body (Simões et al. 2014).

Surface body temperature changes can be used to monitor the physiological processes that occur during oestrus (Soerensen & Pedersen 2015). The use of temperature monitoring for predicting the optimal insemination timing has several benefits over traditional oestrus detection (Firk et al. 2002). Visual observation of sexual behaviour is subjective and as such, instructing stock-people on the correct method has been challenging for commercial producers (Talukder et al. 2014). As temperature is quantifiable and objective, this can be a marker that enables early detection by stock-people to ensure that actions can be implemented in a timely manner (Simões et al. 2014). Additionally, temperature can be measured quickly with a non-invasive device which reduces distress associated with unnecessary handling of animals (Scolari 2010).

Detection of temperature changes has been used as an early marker for oestrus in several species (Bressers et al. 1994; Sykes et al. 2012; Talukder et al. 2014). An increase in the temperature of the vulva from dioestrus to oestrus has been found in cattle (Osawa et al.

2004) and pigs (Sykes et al. 2012). These changes occur because of the associated rise in oestrogen secretion, which causes an increase in blood flow to the reproductive tract, leading to swelling and redness of the vulva (Soede et al. 2011). Greater blood supply results in an elevation in the temperature of the vulva (Scolari et al. 2011).

A decrease in body temperature has been identified immediately prior to ovulation in sows (Scolari et al. 2011). This could be due to the increase in progesterone that occurs alongside ovulation and leads to suppression of the effects of oestrogen (Soede et al. 2011). These observations indicate that skin temperature could be a suitable marker for predicting the correct time for insemination (Luño et al. 2013; Scolari et al. 2011; Simões et al. 2014; Sykes et al. 2012). However, some studies have shown that increasing body temperature is correlated with ovulation and the associated release of luteinising hormone in beef cattle (Piccine et al. 2003) and sows (Luño et al. 2013; Soede et al. 1997). These contradictory results indicate that the use of thermography to predict the optimum time for insemination requires further refinement before commercial application is viable (Weng 2019). The accuracy of temperature monitoring for oestrus detection may be enhanced by choosing specific body locations and using a precise thermometer device.

Fluctuations in skin temperature often occur at different levels based on the site of measurement (Godyń & Herbut 2017). Each surface has different normal temperature ranges and thermoregulation qualities (Green et al. 2008; Schmidt et al. 2013). For example, body temperature is often more stable in the centre of the torso while distal anatomical locations have lower baseline temperatures with a greater fluctuating range due to reliance on the blood flow circulation to maintain temperature (Green et al. 2008). In addition, as pigs do not have sweat glands, they rely on circulating blood to release excess heat in the peripheral limbs which leads to greater variation in skin temperature in these areas (Schmidt et al. 2013). As demonstrated above, the vulva appears to be the most suitable location for monitoring oestrus-associated temperature fluctuations due to effect of oestrogen secretion on blood flow to the reproductive tract (Simões 2012). An alternative location for monitoring skin temperature changes during oestrus is the ear (Bressers et al. 1994; Luño et al. 2013). Surface body temperature monitoring of the ear allowed detection of parturition by identifying an increase in mean ear temperature at 6-12 hours before onset using a radio-telo-metric device implanted

under the skin (Bressers et al. 1994). The temperature of the ear may be an effective measure because the ear is located in close proximity to the brain. The temperature of the brain increases due to the increased blood flow required to sustain a functional nervous system during vigorous events such as parturition or oestrus (Baker 1982). These body locations need to be compared to determine the surface that undergoes the most obvious, consistent and reliable oestrus-associated change in temperature.

Non-invasive technologies reduce distress in animals by removing the need for restraint and minimising the interaction between animals and stock-people (Soerensen & Pedersen 2015). There are several non-invasive tools available for measuring body temperature. Handheld infrared thermometer guns are simple, commercially available tools (Schmidt et al. 2013). Infrared hand-guns utilise a point-and-shoot method to record temperature at a single location with recordings read immediately from the device (Scolari et al. 2011). This technology is relatively inexpensive and requires minimal training but often provides temperature readings with greater variation and reduced repeatability (Jara et al. 2016). Thermal imaging cameras utilise the same technology while providing more advanced analysis options (Sykes et al. 2012; Talukder et al. 2014). These thermometers are more expensive than a handheld device as they enable recording, monitoring, quantification and exploitation of temperature data (Scolari et al. 2011). These devices enable further analysis with external software and can be implemented into automated systems for detection of specific target temperatures or specific threshold requirements (Soerensen & Pedersen 2015). While subdermal thermometer implants have been developed, measurement of temperature using these devices is used sparingly due to the invasiveness of implantation and the associated discomfort in sows, and the possibility of carcase condemnation due to contamination (Jara et al. 2016; Miura et al. 2017). As there is a preference for non-invasive, handheld devices, the infrared thermometer gun and thermal imaging are the most viable technologies for measuring oestrus-associated temperature changes (Godyń & Herbut 2017; Simões et al. 2014).

This study aims to monitor the changes that occur to body temperature at several locations on the ear and vulva during an oestrus event to identify if thermography can be used to predict the optimum timing of insemination. Additionally, these oestrus-associated temperature changes will be monitored using a handheld infrared thermometer gun and a

thermal imaging camera to determine the most effective device for monitoring the body temperature.

4.2 Materials and Methods

4.2.1 Animal Management

The methods involved in this study were approved by the Animal Ethics Committee at The University of Sydney (2013/5942). Data were collected from Large White, Landrace and Duroc crossed sows (n=72) at The University of Sydney Mayfarm piggery in Cobbitty, New South Wales, Australia. The study was conducted over a two-year period from November 2014 to August 2016. Sows were batch-housed in large deep litter pens in groups prior to farrowing in farrowing crates and provided with water and an ad libitum high energy ration. They were moved from farrowing crates to group housing after weaning in groups of 4-6 sows with a pen size of approximately 14 m^2. Body condition score was recorded on exit from the farrowing house. Sows were housed in indoor pens throughout the entire project to enable measurement of ambient environmental temperatures and to prevent outdoor temperatures from causing large fluctuations in the recordings.

4.2.2 Oestrus monitoring

The sows were weaned and underwent a natural oestrous cycle. Oestrus monitoring occurred from three days post-weaning until two days after the last sign of behavioural oestrus. Sows were monitored twice daily (07:00 and 15:00) for signs of behavioural oestrus by conducting a back-pressure test with fence-line boar contact in a designated mating area. Standing heat was defined as the presence of an immobile, standing posture. The onset of behavioural oestrus was defined as the halfway point between the first instance of standing heat and last instance without standing heat. The end of behavioural oestrus was determined by identifying the halfway point between the last instance of standing heat and the first instance without the presence of standing heat. The estimated time of ovulation was defined as the point 66% of the time through the total behavioural oestrus length. The time of predicted ovulation was defined as the time point when peak progesterone occurred in faeces minus 30 hours as demonstrated by Snoj et al. (1998).

4.2.3 Thermal and infrared temperature monitoring

Temperature data were recorded from three days post weaning and continued twice daily (07:00 and 15:00) until two days after the last sign of behavioural oestrus (Figure 4.1). Temperature was recorded using a FLIR thermal camera (FLIR Sys-tems, Inc., Boston, MA, USA) and a handheld infrared thermometer (Fluke 63 Mini, Mektronics, Australia). Both devices were held perpendicular to the body at a distance of one metre from the skin surface for all measurements. The body locations measured were the forehead, the dorsal tip, ventral tip and internal canal of the ear, the left and right pin-bones and the ventral tip, medial surface and internal surface of the vulva (Figure 4.2).

When using the infrared thermometer, the laser pointer was directed at each body location and the temperature was recorded in real-time. Thermal recording involved taking a photograph of the skin surface for assessment at a later time. Images from each of the body locations were uploaded using Thermo-CAM Research Professional 2.7 software (FLIR Sys-tems, Inc.). A single spot temperature was calculated for the same location used with the infrared gun. Rectal temperatures were recorded using a digital thermometer (Omron MC-343F, Kyoto, Japan) and ambient temperatures were collected using a hygrometer (HTC-1). The temperatures recorded during oestrus were subtracted from the baseline levels from 3 days prior to oestrus onset to standardise the values based on normal body temperature for each individual sow, as described by Kessel et al. (2010). The change incorporated an adjusted body temperature by reducing the body temperature value by the difference in the average expected increase in body temperature based on each incremental increase in ambient temperature. This meant that there was a reduction in the adjusted body temperature from the true body temperature at each time-point that had elevated ambient temperatures above 22°C.

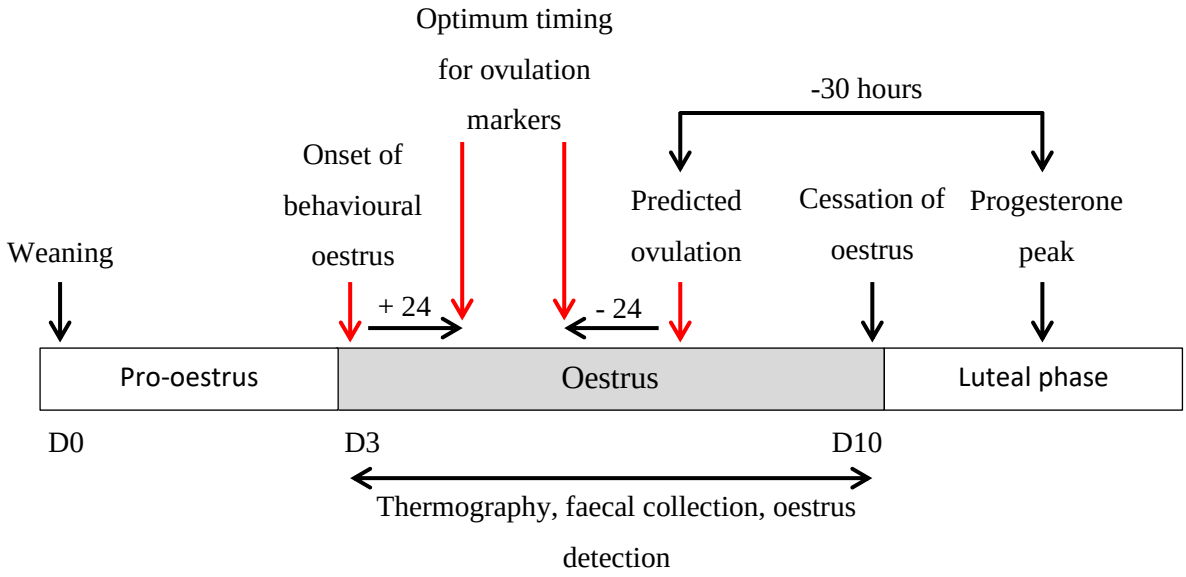

Figure 4.1 Schematic showing the estimated and predicted timings during oestrus in sows to identify if thermal or infrared body temperature is an effective physiological marker of the optimum insemination timing.

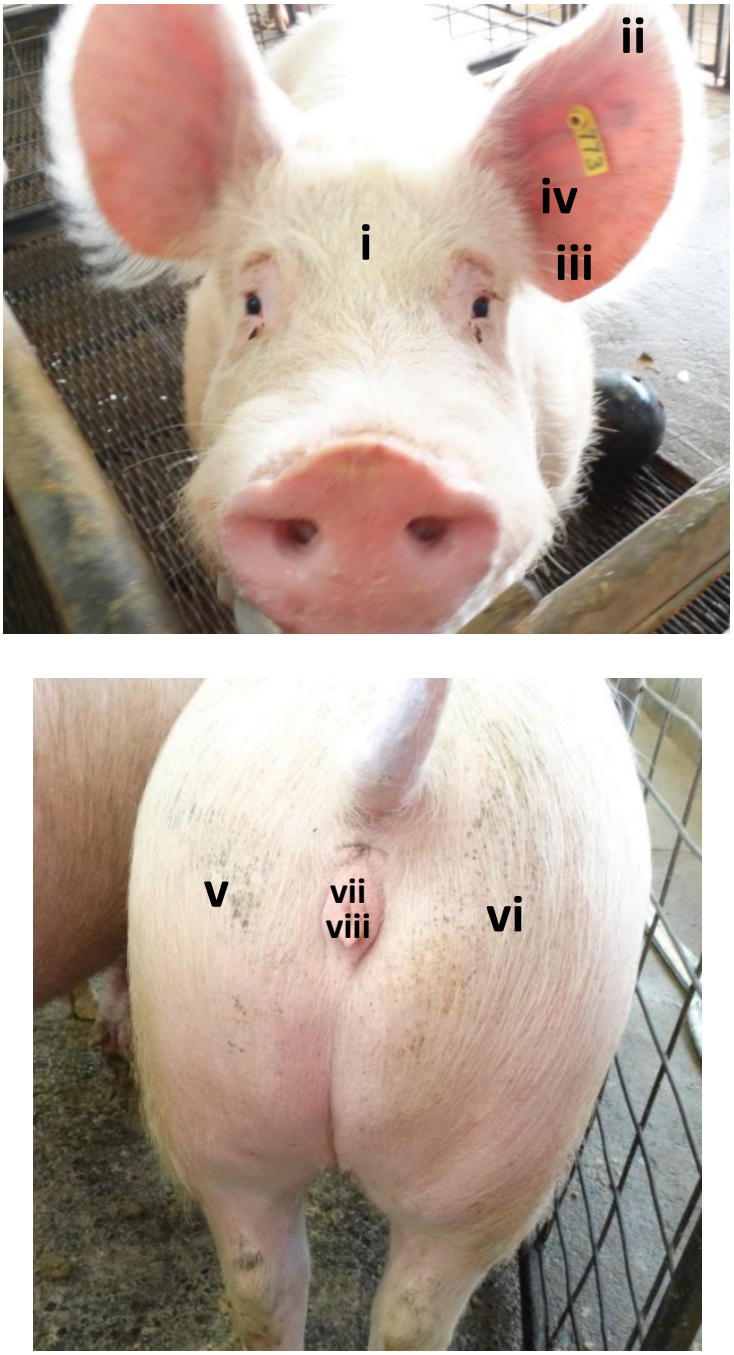

Figure 4.2 Photographs of the specific body locations used in temperature monitoring. Anterior perspective of the pig's head indicating the specific positions for measuring temperature on the **A** face and ears, **i)** centre of the frontal plane of the forehead, **ii)** caudo-dorsal surface of the tip of the ear, **iii)** caudo-ventral surface of the base of the ear, **iv)** internal ear canal on the medial-most point of the ear. **B** hindquarters of the pig indicating specific positions for measuring temperature on the vulva, including **v)** left ischial tuberosity bone (otherwise known as the pin-

bone), **vi)** right ischial tuberosity bone, **vii)** central-medial point of the vulva measured both on the external surface and the internal lining, **viii)** ventral-most point of the vulva.

4.2.4 Faecal progesterone assays

Faecal samples were collected via rectal palpation from each sow and stored at -80°C until further processing. Each sample was dried at 65°C overnight or until dried through. The samples were crushed into a fine dust and large contaminants such as straw were removed. Samples were extracted using ethanol (Sigma-Aldrich Castle Hill Sydney, Australia; 24102-1L-R, >99.8%) at a dilution of 1 mL/0.1 g. All samples were mixed overnight and centrifuged at 800 x g for 20 min. A 500 µL volume of supernatant was transferred to a clean tube for evaporation in a SpeedVac vacuum oven for 18 h at approximately 50°C. The dried extracted samples were frozen and stored at -20°C in a desiccator until use.

Extracted samples were mixed with 100 µL of ethanol followed by 400 µL of assay buffer (1:5 dilution of AB concentrate:deionised water). The samples were vortexed for one minute and allowed to sit at room temperature for 5 min. This process was repeated three times. The ethanol-AB mixture was diluted with 1-5 mL of AB. The reconstituted diluted 50 µL samples were run in duplicate. A 25 µL volume of progesterone conjugate (DetectX® progesterone-peroxidase conjugate in stabilising solution, Arbor Assays) and 25 µL volume of progesterone antibody (DetectX® mouse progesterone specific monoclonal antibody, Arbor Assays) were added to each well and mixed thoroughly. A plate shaker was used to mix the contents of the wells for 2 h. The plate was aspirated five times with wash buffer (1:20 dilution of wash buffer concentrate:deionised water) before addition of 100 µL of TMB substrate (3,3',5,5' - tetramethylbenzidine, Arbor Assays) to each well. The samples were incubated for a further 30 min before adding 50 µL of stop solution. The optical density of each sample was recorded using a plate reader (4PLC software) at a wavelength of 450 nm. The progesterone concentration was calculated based on a standard curve using 4PLC software.

4.2.5 Statistical Analysis

Statistical analyses of the data were completed using the R statistical software package (i386, v3.4.2, 15th Edition, R Core Team). A REML Linear Mixed Model was used to analyse the correlation between progesterone concentration and the various body temperature measurements as well as the correlation between the thermal and infrared temperatures. Temperature data were adjusted for ambient temperature as described in Kessel et al. (2010). A level of $P < 0.05$ indicated a statistically significant result for all tests.

4.3 Results

Of the 72 sows monitored during an instance of heat, 65 sows displayed oestrus and were monitored to enable temperature recording. Of the 65 sows that underwent an oestrous cycle, an ovulation event was predicted in 48 using faecal progesterone concentrations. Reproductive data for the sows examined are shown in Table 4.1. The fluctuations in body temperature occurring at each time point are presented in (Table 4.2) for the thermal imaging camera. Thermal imaging cameras detected no differences in raw temperatures at any of the body locations across the time-points prior to adjustment for ambient temperature. Following the adjustments adapted from Kessel et al. (2010), the external vulva was the only location that underwent an observable change in the 24-hour pre-ovulation period. There was a reduction in vulva temperature during the interval between behavioural oestrus onset and the 24-hour time-point prior to predicted ovulation ($P=0.032$). Additionally, there was a trend towards a decrease in internal vulva temperature at the onset of behavioural oestrus ($P=0.103$) but this was not significant.

Table 4.1 Reproductive data from sows that were used for temperature monitoring during oestrus (mean ± standard deviation).

Sow data	Mean ± SD	Range
Sample size	65	
Parity	3.2 ± 1.2	2-4
Body condition score (BCS)	3.3 ± 0.2	2.5-3.5
Wean-oestrus interval (WEI) days	4.8 ± 1.7	3-7
Length of behavioural oestrus (h)	60.5 ± 10.7	49-66
Estimated time of ovulation (h after the start of oestrus)	34.5 ± 5.1	32-43

Table 4.2 Mean adjusted body temperature measured using a thermal imaging camera at 24 h prior to the first instance of behavioural oestrus, the first instance of standing heat in response to back pressure, 24 h prior to predicted ovulation and the time of predicted ovulation.

Biological marker	24 hours prior to behavioural oestrus	Onset of behavioural oestrus	24 hours prior to predicted ovulation	Predicted time of ovulation (30 h prior to progesterone peak)
Forehead temperature (°C)	27.76 ±0.63	26.19 ± 0.84	27.61 ± 0.79	27.44 ± 0.80
Ear tip temperature (°C)	25.27 ± 1.09	22.74 ± 1.30	24.33 ± 1.17	23.90 ± 1.29
Ear base temperature (°C)	29.80 ± 0.58	28.03 ± 0.71	29.38 ± 0.60	29.00 ± 0.73
Ear canal temperature (°C)	32.16 ± 0.47	30.91 ± 0.58	32.00 ± 0.54	31.81 ± 0.56
Vulva base temperature (°C)	29.80 ± 0.73	27.44 ± 0.52	28.35 ± 0.54	27.83 ± 0.80
External vulva temperature (°C)	31.23 ± 0.39[a]	31.10 ± 0.51[a]	29.68 ± 0.50[b]	29.01 ± 0.70[b]
Internal vulva temperature (°C)	29.03 ± 0.55	27.77 ± 0.56	28.38 ± 0.56	29.01 ±0.73
Left pin-bone temperature (°C)	28.76 ± 0.56	27.07 ± 0.63	27.99 ± 0.59	27.59 ± 0.83
Right pin-bone temperature (°C)	28.82 ± 0.54	26.91 ± 0.68	27.93 ± 0.62	27.30 ± 0.88

Data presented as mean ± S.E.M [a,b] Within the same row, values with different superscripts differ significantly

The use of handheld infrared thermometer guns and thermal imaging cameras gave similar results at eight of the nine body locations (Table 4.3). The external vulva temperature was significantly lower in the immediate from the onset of behavioural oestrus to 24 hours prior to predicted ovulation (P=0.021).

Thermally detected temperatures tended to provide higher values than those observed using handheld thermometer guns at the vulva base. While there were no further significant differences between the two thermometer types, the variation in measurements when using the less sophisticated handheld gun were considerably larger.

Table 4.3 Mean adjusted body temperature measured using a thermal imaging camera and infrared thermometer 24 h prior to the time of predicted ovulation.

	Thermal temperature			Infrared temp			
Biological marker	**Mean**	**Range**	**Stdev**	**Mean**	**Range**	**Stdev**	**P value**
Forehead temperature (°C)	28.79	22-34	2.8	25	16-40	2.6	NS
Ear tip temperature (°C)	28.76	19-34	3.2	25	18-39	3.9	NS
Ear base temperature (°C)	31.69	25-39	2.6	31	22-39	2.5	NS
Ear canal temperature (°C)	31.05	24-37	3.1	30	25-40	2.8	NS
Vulva base temperature (°C)	32.56	22-37	2.3	28	21-39	3.7	P=0.054
External vulva temperature (°C)	32.58	16-37	2.5	32	23-40	3.4	NS
Internal vulva temperature (°C)	35.65	26-40	2.8	34	18-37	3.2	NS
Left pin-bone temperature (°C)	30.84	24-36	3.3	30	19-38	3.7	NS
Right pin-bone temperature (°C)	30.78	25-40	3.0	30	21-38	3.4	NS

4.4 Discussion

The most pertinent result from this study was the significant decrease in temperature from the period after the onset of oestrus to the time-point 24 h prior to ovulation. This is particularly useful for advising the optimal timing for insemination, particularly when combined with observations of behavioural oestrus. The combination of a suite of observational tests to accurately and precisely identify the optimum insemination timing will provide a more robust test for oestrus status. It is recommended that once oestrus behaviour is observed using

the back-pressure test, the temperature of the external vulva should be measured to detect a decrease in temperature. Once the decrease in temperature is detected, insemination should be performed immediately to ensure timely fertilisation. Further studies investigating the hourly temperature profile during this critical period would identify more precisely when the decrease in temperature occurs.

The current study supported the hypothesis that each body location has different amounts of natural variation in temperature (Piccione et al. 2003; Schmidt et al. 2013). For example, the range of temperatures recorded on the forehead was much larger than for vulva locations. The physiological change that is occurring in the body causes varied levels of temperature fluctuation (Godyń & Herbut 2017). Cows experiencing lameness have been found to produce localised temperature increases in the feet or legs (Alsaaod & Büscher 2012; Amezcua et al. 2014) while fever in pigs causes more widespread, systemic temperature spikes as the entire body responds to an immune challenge (Loughmiller et al. 2001; Soerensen & Pedersen 2015). There has been success in the application of IRT programs for detecting systemic changes to the core body temperature, usually associated with deterioration in overall body condition or illness (McManus et al. 2016; Soerensen and Pedersen 2015). However, localised alterations in body temperature to small areas such as the vulva are significantly more difficult to detect with accuracy (Weng 2019). As the inflammation of the reproductive tract occurs in response to oestrogen secretion, there must be close association between the capillaries that transfer blood to the reproductive tissues to cause redness, swelling, and the increase in temperature (Bischof et al. 1995). This could be one reason for the difficulty in detecting temperature changes in the localised regions of the ear or the vulva with sufficient accuracy.

The nine body locations that were assessed for temperature fluctuations exhibited significant variation hence indicating a poor capacity to detect an oestrus event. The external vulva temperature was reduced in the period immediately prior to ovulation, a result which is supported by previous studies (Schmidt et al. 2013; Scolari et al. 2011; Simões 2012). As a result of this study, it can be concluded that the reproductive tissues undergo temperature fluctuations as a result of increased ovarian hormone secretion which leads to vasodilation in the vulva and an increased supply of blood to the reproductive tract (Czaja & Butera 1986).

There were no other significant differences in individual locations over the four time-points indicating that temperature is a highly variable measure that undergoes frequent temporal changes that are not detectable by twice daily observations (Godyń et al. 2019). These results concur with those of a previous study in cows by Talukder et al. (2015), where vulva temperatures enabled detection of oestrus in approximately 80% of cows. However, in that study 21% of these oestrus-detected cows were subsequently identified as false positives. These outcomes prevent widespread implementation of handheld infrared devices into commercial production as high rates of false positive oestrus detection are not acceptable (Scolari et al. 2011). As the purpose of quantifiable data is to improve upon the conception rates currently obtained using behavioural-based oestrus detection, the use of IRT cannot be recommended for commercial application when using the raw data alone (Nejad et al. 2019).

Another possible explanation for the variability in vulva temperature across individuals is parity, as age impacts on the physiological composition of the reproductive tract. High parity sows have undergone several farrowing events that can lead to injuries to the vulva and internal reproductive tract, scarring and irreparable damage (Kaiser et al. 2018). This damage can affect the capillaries in the external reproductive tract leading to impaired blood flow (Steihler et al. 2015). This indicates that there needs to be consideration of any vulva injuries in older parity sows when this temperature is being used as a predictor of insemination timing.

Ambient temperature contributes to fluctuations in body temperature by causing changes to the thermal environment, especially when the skin surface is the target location (Dai et al. 2017). IRT data can be enhanced by implementing adjustments based on ambient temperature conditions (Soerensen & Pedersen 2015). The current study conducted simple modifications to determine if these results could be useful for the detection of oestrus in sows. To overcome the variability in body temperature, use of a simple equation that would allow body temperature to be adjusted based on ambient environmental temperature could facilitate commercial application (Talukder et al. 2014). The temperatures recorded in this trial indicate that the external surface of the vulva undergoes significant changes.

This study was the first to compare the effectiveness of thermal imaging cameras with handheld infrared thermometer guns. Temperatures obtained from infrared thermometers

resulted in the greatest level of variation. The use of handheld guns for detecting the optimum timing of insemination has rarely been examined, perhaps due to the unpredictable equipment which leads to low accuracy readings (Soerensen & Pedersen 2015). While these devices are simple and easy to implement, the requirement to conduct alterations to the raw temperature values will impede successful use of this technique for detecting oestrus. Testing and verification of any device is required prior to deeming it suitable for this purpose (Church et al. 2014). The difficulty observed in recording results that are free from ambient noise was problematic in the current study (Kessel et al. 2010). Alternatively, a device that is capable of automatically recording and processing raw temperature data into actionable notifications would be more beneficial (Ibarra-Castanedo et al. 2004). This technique has been successful in cattle under temperature-controlled housing environments which have enabled higher accuracy and precision in thermal surveillances programs (Talukder et al. 2015). The sophisticated forms of IRT thermal imaging cameras enabled more reliable outcomes over handheld infrared guns. Enabling innovative tools is more valuable than struggling to monitor temperature using simple tools that result in ineffectual data measurements.

The effect of oestrogen on reproductive tissues relies on the close association between the circulatory network and the epithelial layer of the internal tract. One potential explanation for the irregularity in body temperature despite the known positive correlation with plasma oestrogen concentration, is that the physiological changes that occur in the reproductive tract are often heterogeneously applied to the large surface area of the tissue itself (Ash & Heap 1975; Frisch 1984). The large surface area of the reproductive tract leads to formation of compartments with less access to the circulating oestrogen resulting in an uneven distribution of heat (Krajewski-Hall et al. 2018). Consistent maintenance of temperature across the entire reproductive tract, including the external surface of the vulva, is impossible. To enable effective detection of temperature change to the reproductive tissue, the use of internalised thermometers is suggested. While there is minimal research focused on implementation of internal thermography in pigs, there has been relative success using this technology for monitoring core body temperature in horses (Green et al. 2008; Green et al. 2005).

4.5 Conclusions

The thermal imaging camera detected a reduction in the external surface temperature of the vulva 24 hours prior to ovulation and could be a useful marker for predicting the optimum timing for inseminations in sows. There were no other body locations that demonstrated a significant fluctuation in the period prior to ovulation. However, the use of simple, handheld infrared guns for the detection of skin temperature changes is probably not viable due to inconsistent and variable outcomes associated with ambient, environmental conditions. Despite the variability, the use of more advanced thermal detectors that may enable more consistent and reliable detection of the vulva temperature change prior to ovulation warrants further investigation.

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Chapter 5

Accelerometer-based quantification of sexual behaviour

Chapter 5

Classifying normal and oestrus behaviour using collar-mounted tri-axial accelerometers in group housed sows

5.1 Introduction

Improved animal management is possible by examining and dissecting complex actions and activities displayed by animals (Marchioro et al. 2011). Manual observation and classification of animal behaviour requires targeted training to enable individual stock-people to develop sufficient intuition-based decision-making skills (Bidder et al. 2014; Marchioro et al. 2011). Visually differentiating between behaviours can be time-consuming and labour intensive for stock-people and as such this process is often neglected or allocated less labour time than is required (Cornou et al. 2011). This is particularly relevant as commercial pig production becomes more intensive with larger herd sizes that lead to difficulty in accessing and providing adequate care for all animals individually (Cornou 2006; Crump et al. 2018). A more thorough understanding of behaviour through the use of innovative, insightful technology can improve animal welfare (Ruiz-Garcia et al. 2009).

One example of technology that can be used to exploit intricate farm processes and behaviours is through digital data recording devices (Fogarty et al. 2018; Wathes et al. 2008). With current technology, it is now realistic to quantify animal behaviour which was formerly impossible because of the subjectivity and inconsistency between individual stock-people (Norton et al. 2019; Banhazi & Black 2009). If common behaviours such as walking, eating and lying can be identified with ease and accuracy, it is possible to recognise altered behaviours that might suggest the presence of disruptions (Alvarenga et al. 2016). Detection of these behaviours can permit specific management practices to be conducted to rectify issues such as disease or to determine animals that require additional assistance during oestrus (Cornou 2006), and parturition (Oczak et al. 2015). In addition, these devices have the potential to be adapted to enable automatic identification of these behaviours which is beneficial for labour

conservation and time management on commercial farms (Dobos et al. 2014; Norton et al. 2019).

There are a range of technologies that can distinguish between different behaviours including infrared photocells which use light beams to detect posture (Jones 1966), time lapse video footage to detect behaviour lengths (Bressers et al.1991), electronic monitoring systems to track locations and social interactions (Paolucci et al. 2008) and pedometers which track activity levels (Tani et al. 2013). These technologies can identify a single behaviour but have not been able to successfully distinguish between multiple behaviours (Ruiz-Garcia et al. 2009). An alternative technology for classifying behaviours is tri-axial microelectromechanical system (MEMS) accelerometers (Alvarenga et al. 2016). This technology collects orientation data from three axes of movement at infinitesimal intervals in order to develop unique acceleration signal profile over a period of time for each behaviour (Cornou et al. 2008). Acceleration signals can produce a digital expression of the behaviour changes exhibited by animals (Brown et al. 2013). These technologies have the potential to enhance current manual detection and classification procedures for behaviours in commercial situations by enabling recognition of these behaviours (Alvarenga et al. 2016; Marchioro et al. 2011).

The behaviour of a sow can be linked to the status of that animal (Wemelsfelder 2007). Individual classification of animals can occur by determining the animals' status in terms of health, reproduction and welfare, amongst other things (Neethirajan 2017; Rutten et al. 2013). Previous studies have classified normal, daily occurring behaviours such as walking, feeding and resting through the use of unique acceleration signal profiles (Cornou & Lundbye-Christensen 2008; Cornou & Lundbye-Christensen 2010; Escalante et al. 2013). These behaviours occur regularly with the presence of these signals indicating a healthy status. Deviation from normal patterns within an acceleration signal profile can indicate a change in the animals' status (Matthews et al. 2016). From a breeding management perspective, sows undergoing oestrus often demonstrate elevated activity levels as the individual attempts to actively seek a mate (Oliviero et al. 2008). The behaviours that are involved in this period of heightened activity should be quantified and this change in activity could be used as a marker for identifying the oestrus event and the associated optimum timing for insemination

(Labrecque & Rivest 2018). Stock-people would be able to search for a signal profile change that represents a specific behaviour associated with oestrus, classify the reproductive status of the animal and be able to conduct inseminations at the required time to maximise conception rates (Saint-Dizier & Chastant-Maillard 2012). Farrowing activity has been monitored in sows using accelerometers and was found to produce a unique signal profile that was observed in 75-100% of sows (Cornou et al. 2011). While activity levels during oestrus have been assessed using such technologies, the signal profiles associated with more specific sexual oestrus behaviours have not been identified.

This study first aimed to assess the impact of collar-based accelerometers on normal behaviour in group housed sows. Subsequent examinations attempted to categorise and quantify the main behaviours that are observed in group housed sows during oestrus using tri-axial MEMS accelerometers. The overall aim was to identify unique signal profiles that would enable differentiation between normal daily activities and sexual behaviours and to determine the specificity, sensitivity, accuracy and precision of the accelerometers when detecting these signals. The behaviours examined were standing, lying, walking, eating, flank nosing, sow-to-sow mounting, urogenital sniffing and standing heat across four different blocks of time (3, 5, 10 and 15 second periods).

5.2 Materials and Methods

5.2.1 Animal Management

The methods involved in this study were approved by the Animal Ethics Committee at The University of Sydney (2015/862). Data was collected from Large White, Landrace and Duroc crossed sows (n=24) at The University of Sydney Mayfarm piggery in Cobbitty. Sows were housed within cement-based pens and housed in groups of 4-6 sows based on a pen size of approximately 14 m^2. The sows had ad libitum access to water and a dry sow high energy ration. The average parity of the sows included in the study was 3.4±1.25 and the average weight was 242±65 kg.

5.2.2 Experimental design

The study was split into two parts. Part one investigated if attachment of collar-based accelerometer systems affected the ability of the sows to perform normal behaviours. Part two investigated the monitoring and annotation of several behaviours during oestrus to enable quantified accelerometer signal profiles to be identified for each action (Figure 5.1).

5.2.3 Accelerometer systems

The accelerometer system contained a tri-axial microelectromechanical systems (MEMS) data motion logger (AML V2.0, AerobTec, Bratislava, Slovakia). The accelerometer had a weight of 8 grams, dimensions of 20 x 30 x 7cm and was attached to a high discharge 350 mAh Turnigy nano-tech Lipo battery (Turnigy, Hong Kong). The AerobTec motion logger 2 (AML) system records acceleration as a result of dynamic motion on the sagittal, frontal and transverse planes, which were applied consistently across all sows. The planes of motion were allocated relative to the orientation of an immobile sow standing with legs perpendicular to the ground. The planes were transformed so that the X, Y and Z axes were associated with left-right (sagittal/horizontal), up-down (frontal/vertical) and forward-backward (transverse/swing) motions, respectively. The accelerometer collected data at a rate of 5 Hz resulting in sampling points of 5 measurements per second and a range of ±2g. The system was secured in a sealed ABS (acrylonitrile butadiene styrene) mounting enclosure box (Jaycar, Rydalmere, Australia) and attached to a 4cm wide nylon cattle collar using 1/4 inch zinc plated hex bolts (Zenith, Illinois, USA). The ABS box was positioned on the ventro-medial surface of the neck and secured around the caudal surface of the mandible (Figure 5.2). The AML sensors were standardized by placing them on a flat surface and moving each axis individually to determine baseline signals.

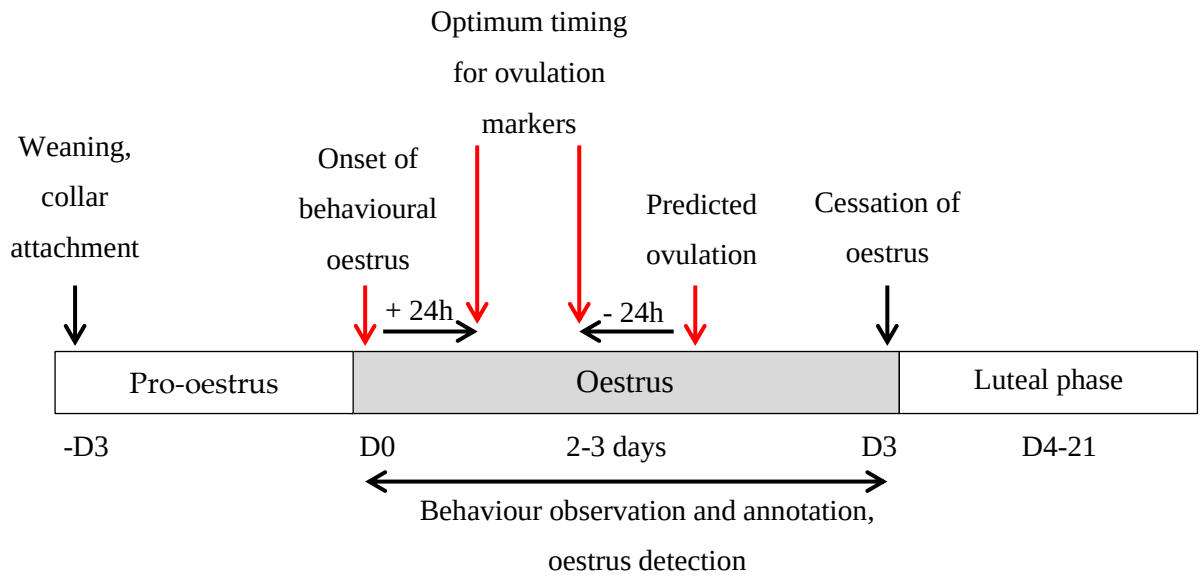


Figure 5.1. Schematic showing the time points during oestrus in sows used to identify the accelerometer detected behaviour.

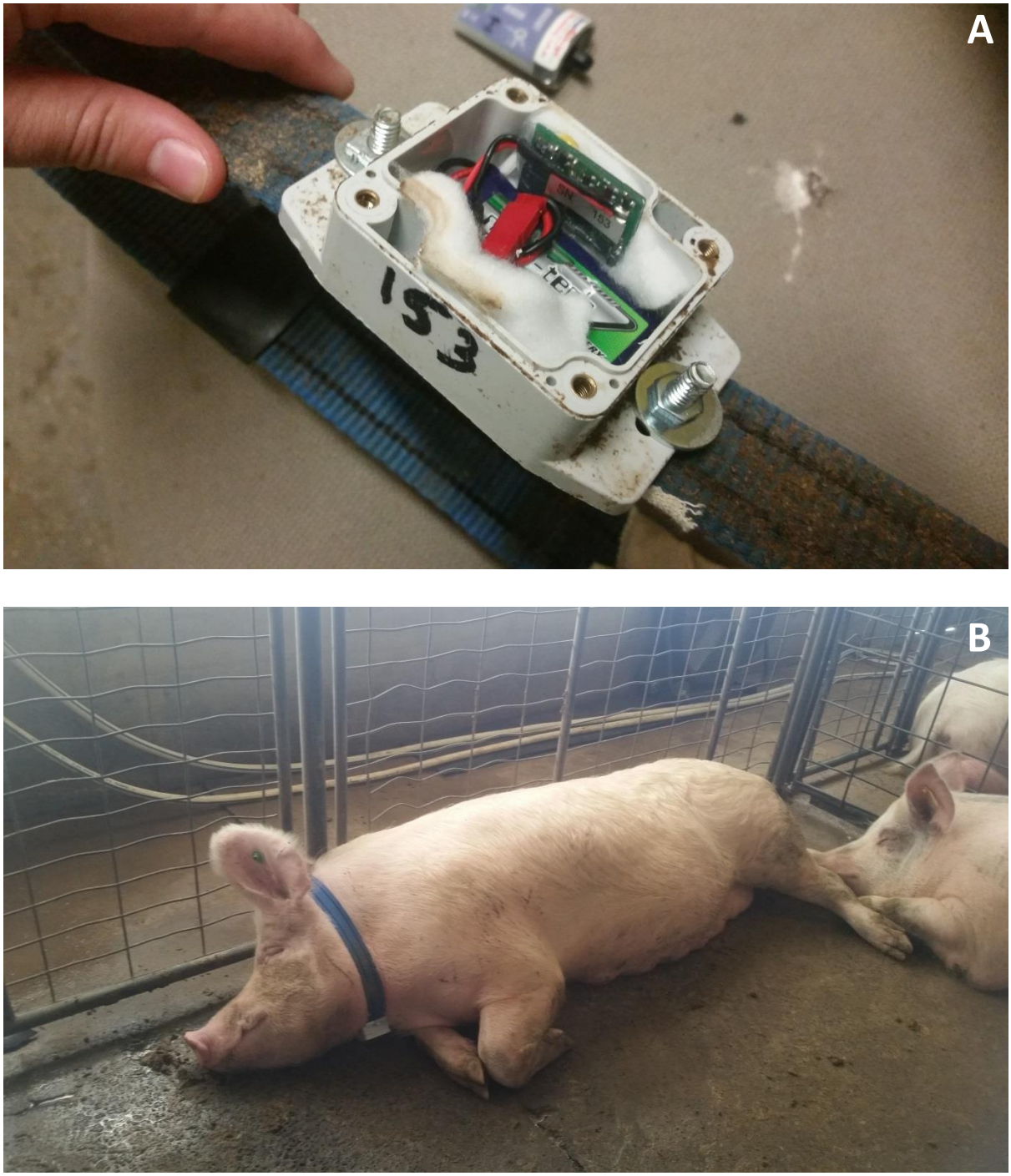


Figure 5.2. Panel A displays the arrangement of the battery and data motion logger inside the enclosure box mounted on the nylon collar. Panel B displays the placement of the enclosure box, on the ventral surface of the neck, when the collar is worn by the sow.

5.2.3 Part One - Comparison of collared and non-collared behaviour

At 08:00 on Day 1, the sows were randomly allocated to one of two treatments; collared (n=12) or non-collared (n=12). Each sow was allocated a number between 1-24 which was marked on both sides of the rump, the middle of the back and the forehead for visual identification. Once the collars were attached, the animals were moved into deep-litter, straw pens with an area of 28m^2. Each pen was allocated 6 animals from each treatment. Real-time behavioural observations were documented by two technicians using scan sampling at 5-minute intervals between 09:00 and 14:00 for five days (Lehner 1998). The behaviours that were recorded are shown in Table 5.1. The collars remained on the animals throughout the entire experimental period.

Table 5.1. Behaviour ethogram for differentiation of events observed via scan sampling adapted from Manning et al. (2017).

Behaviour state	Behaviour event	Description
Active	Walking	Forward motion with a continuous, repetitive stride while in an upright posture with a pace of one movement of each leg per second.
	Running	Forward motion with a continuous, repetitive stride while in an upright posture with a pace of two or more movements of each leg per second.
Eating	Foraging	Head orientation directed below the plane of the torso and interacting with the surface of the ground. Usually but not limited to being accompanied by small, repetitive, forward thrusts of the nose and occurring while the sow is either stationary or walking.
	Chewing	Repetitive grinding of the jaw.
	Drinking	Direct connection between the sow and a water supply accompanied by a swallowing motion.
Social	Object interaction	Any interaction between the sow's head, face, body or feet with an object for more than one second.
	Self-directed interaction	Any activity directed towards the sow itself including licking, itching or grooming.
	Sow-directed interactions	Any interaction between the sow's head, face, body or feet with another animal for more than one second or any activity directed towards another sow including licking, itching, grooming, fighting and object interaction.
Posture	Stationary	Standing immobile without performing any other defined motions while all four legs remain idle.
	Lying left	Immobile posture while body is lying on the left side.
	Lying right	Immobile posture while body is lying on the right side.
	Lying ventral	Immobile posture while body is lying on the ventral surface with limbs held against the body.

5.2.4 Part Two – Quantification of oestrus behaviours

Sows (n=24) were fitted with an accelerometer system upon entry to the mating shed, immediately after weaning. These sows were not the same individuals from part one. The sows were moved to cement pens in groups of six with an area of approximately 15m^2. The animals were able to undergo a natural oestrus event following weaning at 21 days. Acceleration data was collected from 24 sows continuously from weaning until the end of the behavioural oestrus, which was classified as the presence of standing heat in response to back pressure. Collars were removed between 12:00-13:00 every second day to download the output produced from the accelerometer to AML software (AerobTec, Bratislava, Slovakia) and replace the battery. Continuous video footage recorded the behaviours displayed by the sows using two automatic video cameras.

5.2.4 Video annotation and quantification of behaviours

The video footage was loaded into CowLog (Version 3.0.2) to undergo manual annotation for identification of the main activities; lying, standing, walking, foraging and oestrus behaviours including flank nosing, sow-to-sow mounting, urogenital sniffing and standing heat. The video was further separated into sub-activities; lying (left side, right side, ventral), standing, walking (slow-pace, fast pace), eating (foraging, chewing, drinking). The timestamp on the video footage and AML signal output were manually synchronised to form a single dataset.

Lying was classified as a motionless, horizontal state that was present with any head position over an extended period. Similarly, standing was classified with the same motionless, horizontal state with an additional extension of the legs. Forward motion with an absence of eating behaviour and an upright head position was classified as walking. Eating was classified as the action of actively searching for food with the head below parallel to the ground. This is inclusive of the shovelling head motion associated with foraging, the resumption of a linear head-body orientation and chewing of food with an upright head orientation. Oestrus was identified by an extended period of sow-sow interaction that involved mounting of one sow by another or both horizontal and/or vertical stimulation of the flank or urogenital areas of other sows (Levis et al. 2011).

The data file obtained from AML sensors were analysed using R (i386 v3.4.2, R Core Team (2017)). The activity data was separated into smaller time periods of 3, 5, 10 and 15 seconds with a total of 15, 25, 50 and 75 individual activity values for each sow, respectively. A range of values were calculated to analyse the raw X, Y and Z signal data in a confusion matrix (Table 5.2). The mean, maximum, minimum and standard deviation from a calculated signal magnitude area (SMA, $|x_i| + |y_i| + |z_i|$) and signal vector magnitude (SVM, $\sqrt{(x_i^2 + y_i^2 + z_i^2)}$) for the X, Y and Z axes were calculated to give a total of 24 values. The data were further analysed by using 50% of the signal profiles as a test data set to develop a suitable model and 50% as a validation data set to identify the accuracy of the model (Alvarenga et al. 2016).

Table 5.2. Definitions of the calculations used within a confusion matrix to assess the effectiveness of accelerometer signal profiles for predicting oestrus behaviours

Measurement	**Captured population**	**Definition**
Accuracy	TP	Number of correct predictions of behaviour using signals
Sensitivity TP/(TP + FN)	FN	Behaviour was observed but incorrect classification occurred using the signal
Specificity TN/(TN + FN)	FP	Behaviour was not observed and incorrectly classified
Precision TP/(TP + FP)	TN	Number of correct predictions of a behaviour being absent

TP: True positive; TN: True negative; FP: False positive; FN: False negative.

5.2.3 Statistical analysis

Statistical significance was determined via a P value of less than 0.05 and all analyses were completed in R (i386 v3.4.2, R Core Team (2017)). The behaviours observed on collared and non-collared sows were analysed using individual REML models. Each model used fixed terms of Sow and both Collar type and Date/Time as nested effects.

The behaviour results were analysed using a random forest in R to identify the five most relevant calculated values. These values were used in a regression-based decision tree to determine if the signal profiles can predict the associated behaviour. A confusion matrix was used to calculate the accuracy, precision, sensitivity and specificity. An analysis of the most effective time periods (3, 5, 10 and 15 sec) to detect behaviour with signals occurred via a confusion matrix.

5.3 Results

An oestrus event was observed in 48 sows and the summary reproductive data is presented in Table 5.3.

Table 5.3. Reproductive data of the cycling sows used for monitoring accelerometer-based quantification of sexual behaviour in sows (mean ± S.E.M).

	Mean ± S.E.M
Sample size	48
Parity	3.4 ± 1.25
Body condition score (BCS)	3.0 ± 0.5
Wean-oestrus interval (WEI)	4.6 ± 1.5
Length of behavioural oestrus (hrs)	62.5 ± 11.4
Estimated ovulation based on behavioural oestrus (hrs)	39.3 ± 4.4

5.3.1 Collared versus non-collared behaviour analysis

There were twelve behaviours monitored across the collared and non-collared animals and the results are summarised in Table 5.4. Walking occurred 3.7 times more frequently in sows that were collared (P = 0.02). Non-collared sows had an average of 18.3 occurrences of chewing detected daily which was significantly more than the 7.1 instances in collared animals (P = 0.04). Left orientated lying postures were more prevalent in collared sows (P = 0.02). The attachment of collars did not impact on the expression of the remaining nine behaviours.

Table 5.4. Mean daily number of occurrences of each behaviour recorded for collared and non-collared sows over a five-day period.

Behaviour	Collared sows	Non-collared sows	S.E	P value
Walking	4.5	0.8	1.6	P = 0.02
Running	0.4	0.8	0.3	P = 0.16
Foraging	6.8	6.75	2.3	P = 0.47
Chewing	7.1	18.3	4.2	P = 0.04
Drinking	0.6	0.5	0.2	P = 0.34
Object interaction	1.6	0.8	0.7	P = 0.50
Self-directed	0.3	0.2	0.2	P = 0.25
Sow-directed	0.5	0.6	0.2	P = 0.22
Stationary	16.3	15.4	12.9	P = 0.28
Lying left	15.2	12.3	1.2	P = 0.02
Lying right	12.9	14.6	1.5	P = 0.07
Lying ventral	7.4	8.8	2.2	P = 0.21

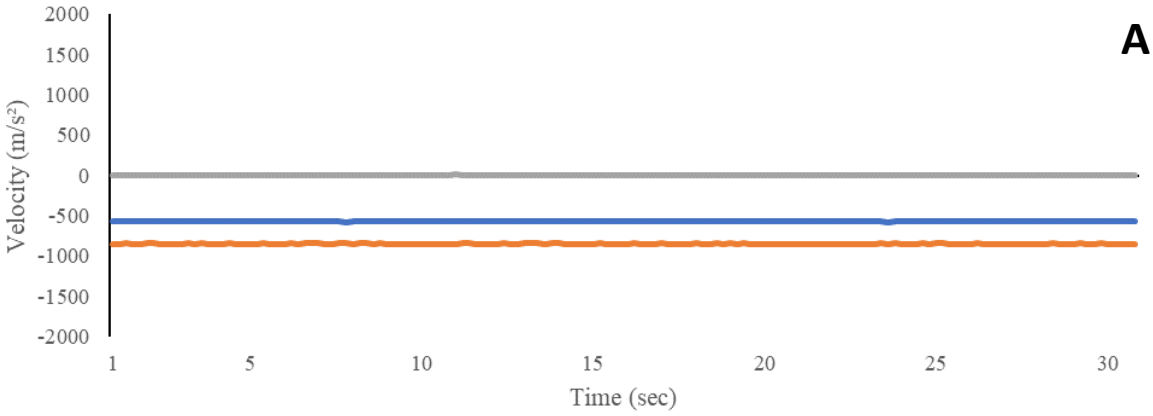
5.3.2 Behaviour classification and quantification

The classification of behaviours was made possible by identifying the signal profiles associated with each action as demonstrated in video footage. The number of instances of each behaviour and the number of sows associated with each instance are shown in Table 5.5.

Table 5.5. The number of instances of each behaviour and the number of sows associated within an epoch.

	Epoch							
	3s		5s		10 s		15 s	
Behaviour	**Number of data points**	**Number of sows**	**Number of data points**	**Number of sows**	**Number of data points**	**Number of sows**	**Number of data points**	**Number of sows**
Lying	3780	14	6300	14	12600	14	18900	14
Standing	4050	15	6750	15	13500	15	20250	15
Walking	3240	12	5400	12	10800	12	16200	12
Eating	3780	14	6300	14	12600	14	18900	14
Oestrus	2700	10	4500	10	9000	10	13500	10

The X, Y and Z axes were associated with a directional movement for horizontal, vertical and swing planes. Representative signal profiles for predominant behaviours are shown in Figure 5.3. Both standing and lying behaviours demonstrated stable, effectively level signal profiles on the horizontal, vertical and swing axes. Differentiation between these two behaviours was achieved by identifying a vertical axis with a greater magnitude than the other axes for standing while lying was demonstrated by a horizontal bias in either the left or right direction. Walking was demonstrated by a more complex change in all three axes, with small magnitude changes in the vertical and swing axes, showing slight head movements forward and pitching downwards respectively, and a moderate horizontal movement from left to right as the legs alternate for the motion of stepping. Running had a similar signal profile but was distinguished by rapid repetition of this behaviour and significantly greater movements in all three axes.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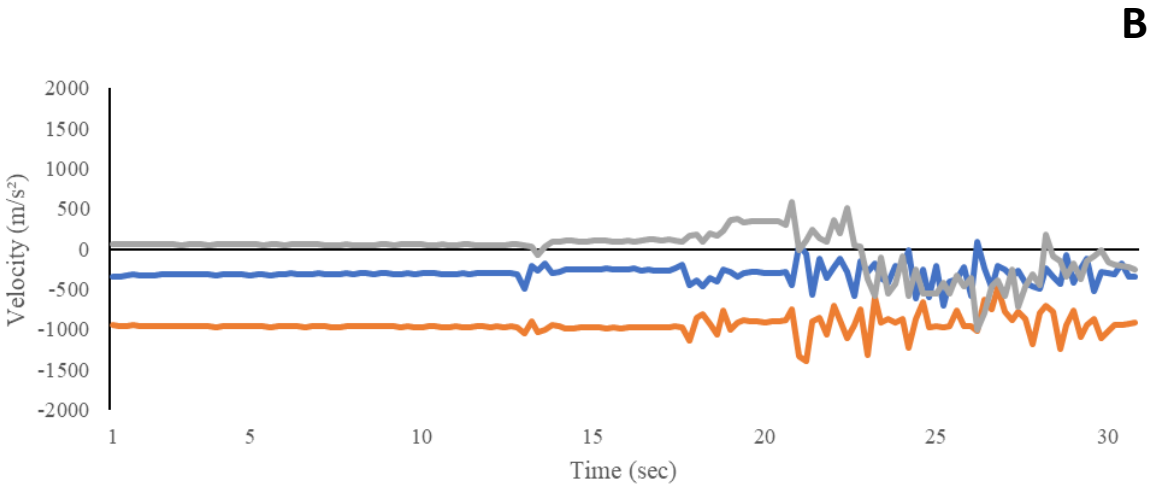
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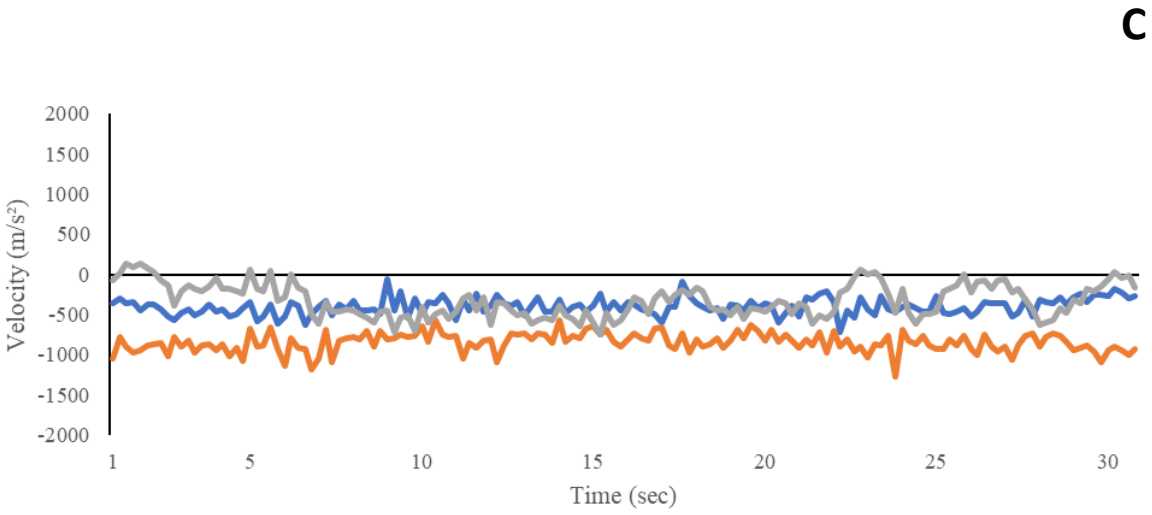
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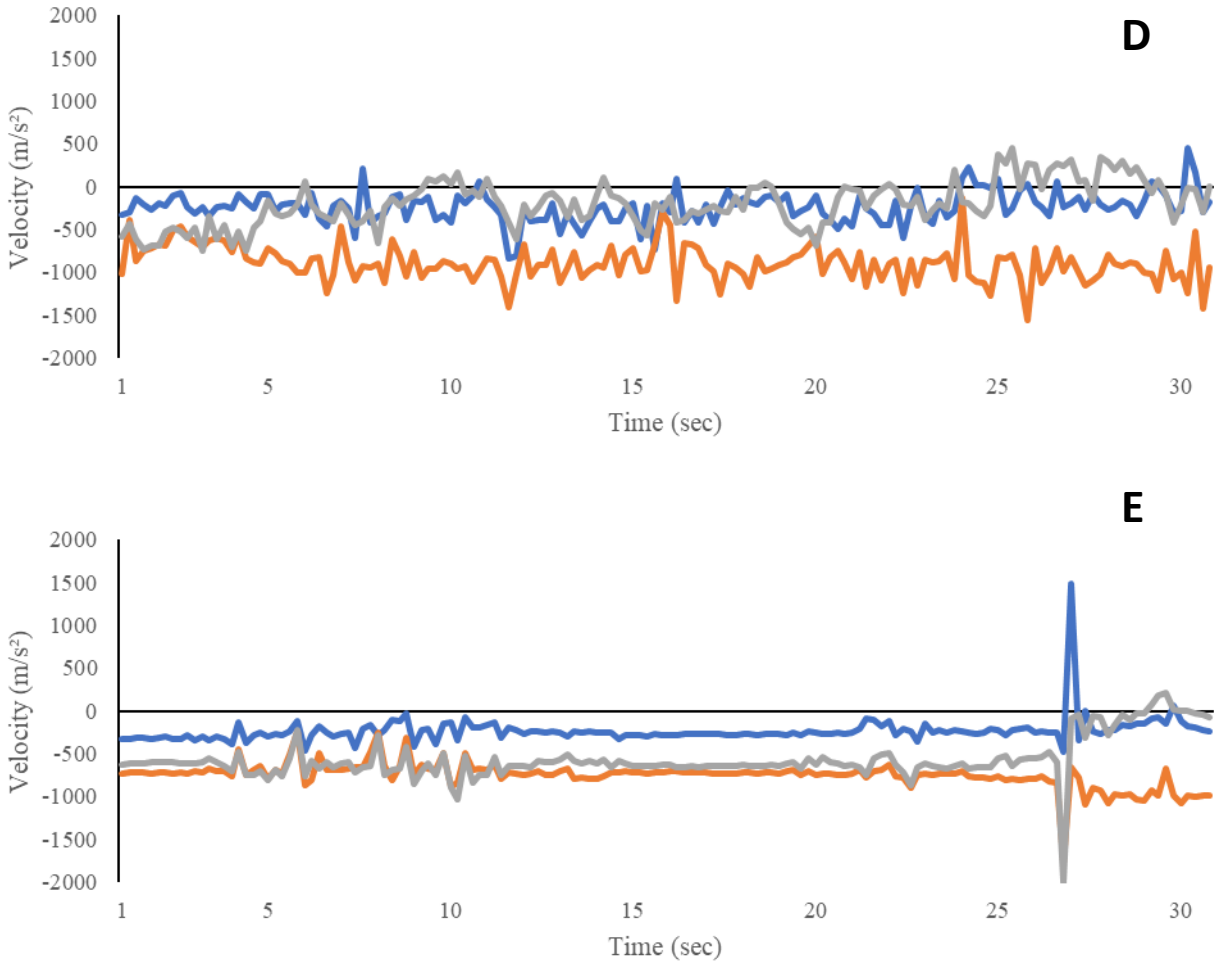

Figure 5.3. Representative raw tri-axial MEMS accelerometer signals obtained over a 6h period from a sow displaying behaviours of **(A)** lying, **(B)** walking, **(C)** eating, **(D)** flank nosing and **(E)** sow-to-sow mounting using a sampling rate of 5 Hz. The X, Y and Z axes correspond to the colours of orange, grey and blue, respectively.

Foraging signal profiles involved periods of activity where the action of searching for and chewing food occurred in repetitive bouts. This was shown by the depression of the head towards the ground with slight horizontal acceleration from left to right as the sow followed the line of sight in an attempt to discover food. These actions were interspersed with small, intense, forward movements in the swing axis that allowed detection of a flicking motion that indicated manipulation of any food or housing substrates. Within this time there were also occurrences of positive vertical movement where the head rose to a resting position parallel to the ground. Small fluctuations in the vertical and horizontal axes occurred during chewing as the sow transferred food throughout her mouth and swallowed.

Oestrus was clearly recognised by the presence of recurrent bouts of activity that was verified by a three-fold increase in acceleration in all three axes (Figure 5.4). This intensification occurred as a result of multiple behaviours, all of which increased the overall level of activity observed in oestrus sows. Flank nosing, which involved a sow stimulating the ventral surface of another sow, was identified by repeated vigorous upward scooping motions with the nose. This action was characterised by high magnitude vertical acceleration and was often repeated several times in short succession. Urogenital sniffing and interaction were identified by the same signal profile, but with a smaller change in magnitude within the vertical axis. Sow-to-sow mounting, detected from the perspective of the sow performing the mounting, was easily discernible by the large increase in vertical acceleration which occurred during the transition from standing on two legs to standing on four legs. The signal profiles for flank nosing and urogenital sniffing behaviours were difficult to separate from feeding and foraging actions due to similarities in the motion involved in both of these activities. However, these oestrus activities of flank nosing, and urogenital interaction occurred in regular, repeated bouts throughout the day. This contrasted with feeding actions, which involved a single daily occurrence, often at the same time of the day when feed was provided.

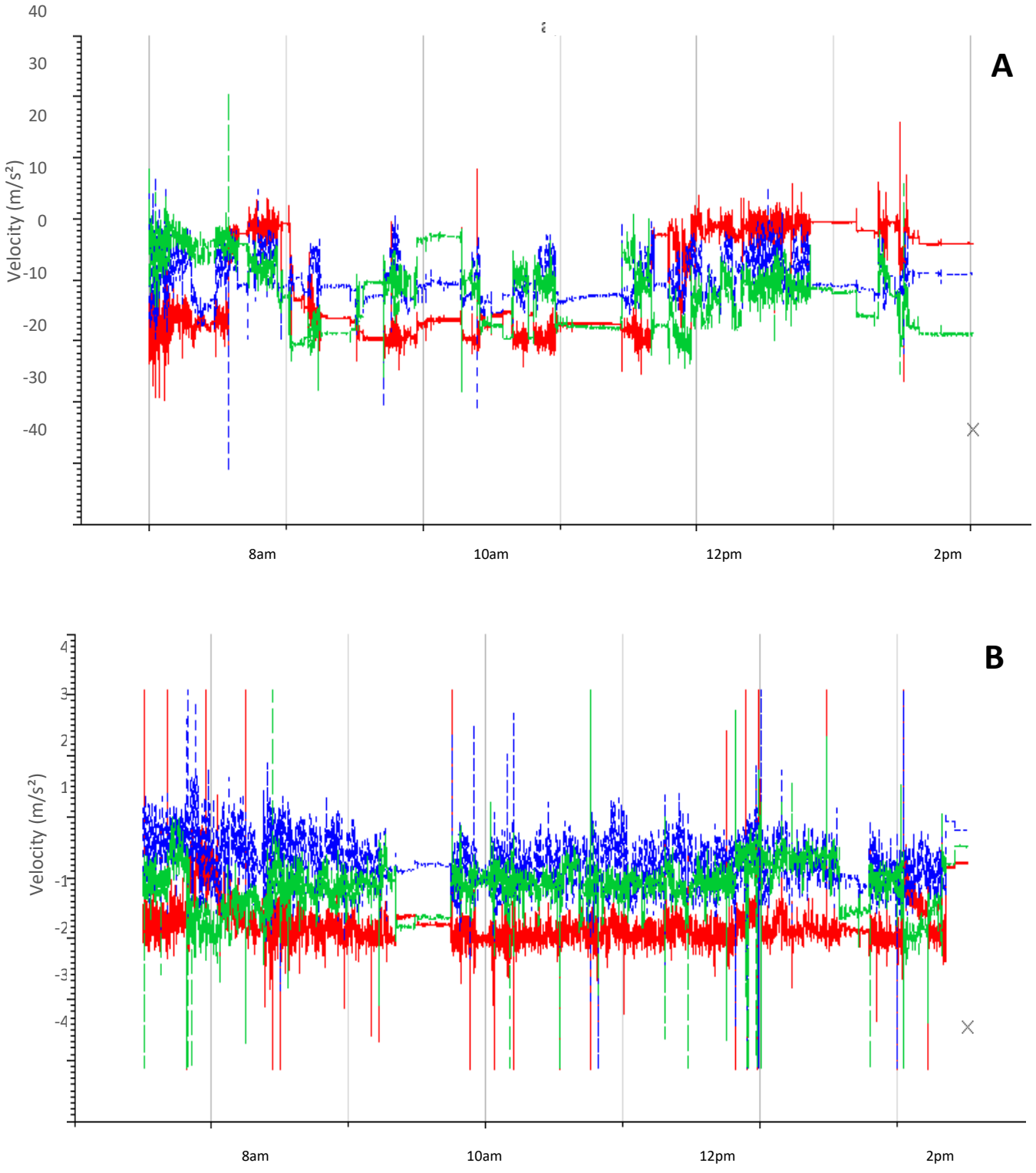

Figure 5.4. Representative raw tri-axial accelerometer signals obtained from sows during **(A)** dioestrus and **(B)** oestrus.

Standing heat was not assessed in a confusion matrix as the behaviour is, by definition, the display of an immobile posture and hence cannot be detected by a change in the signal profile. The decision-tree identified performance outcomes for the three oestrus behaviour markers of flank nosing, sow-to-sow mounting and urogenital sniffing (Table 5.6). For all performance outcomes, the values for flank nosing and sow-to-sow mounting were greater than those for urogenital sniffing. The behaviour with the highest accuracy in the evaluation model was sow-to-sow mounting with 89.5%, while that with the lowest accuracy was urogenital sniffing with 55.3%. Both flank nosing and sow-to-sow mounting involved high precision outcomes while urogenital sniffing behaviours were the lowest behaviour at 57.5%. Sensitivity was highest for sow-to-sow mounting with 94.8% of the evaluation model but this outcome decreased to 85% in the validation model. The specificity of urogenital sniffing decision-making trees was the lowest with 42.8%. The results obtained using the validation data were very similar to those obtained using the evaluation data.

Table 5.6. Decision-tree outcomes for performance percentages of accuracy, precision, sensitivity and specificity for oestrus-related behaviours in sows using the evaluation and validation data sets.

	Flank nosing	Sow-to-sow mounting	Urogenital sniffing
Evaluation			
Accuracy	78.6	89.5	55.3
Precision	81.8	82.4	57.5
Sensitivity	86.4	94.8	62.3
Specificity	88.5	92.6	42.8
Validation			
Accuracy	80.4	90.4	54.8
Precision	83.0	84.5	48.3
Sensitivity	82.1	85.0	64.7
Specificity	89.0	93.4	52.1

5.4 Discussion

This study is the first to classify sexual oestrus behaviours of flank-nosing, mounting and urogenital sniffing using an accelerometer signal profile from sensors mounted on a collar. Raw signal profiles were also obtained for several key daily behaviours including lying, standing, walking and foraging. The cumulative activity level for sows during oestrus was three-fold higher than sows during dioestrus. Detection of an oestrus event was therefore possible through quantification of the cumulative activity index which demonstrates the intensity of behaviours.

Previous studies have identified unique signal profiles that enable detection of behaviour states such as parturition in sows (Cornou et al. 2008) and rumination in sheep (Alvarenga et al. 2016). Other applications for this technology include the detection of

conditions that affect movement, such as lameness (Nalon et al. 2013). The desired outcome when applying these big-data technologies is to enhance the knowledge base of stock-people and to assist the decision-making process to improve production efficiency (Marchioro et al. 2011). The results of the current study show that oestrus behaviours can be quantified to effectively detect sexually receptive sows.

The detection of oestrus behaviours when using accelerometer technologies has had differing rates of success. The accuracy of the visually obvious behaviours of both flank nosing and sow-to-sow mounting were high in this study, with 78.6% and 89.5% of these behaviours correctly classified, respectively. These accuracy outcomes are comparable to those obtained by Cornou et al. (2011) for parturition-associated behaviours. Current oestrus detection using behavioural-based observation methods enables farrowing rates of 70-90% to be achieved on high-performing farms if various other factors including parity, season and stock-person skill level are accounted for (Koketsu et al. 2017). The combination of accelerometer-detected flank-nosing and sow-to-sow mounting may facilitate effective oestrus detection and identification of the optimum timing of insemination (Labrecque & Rivest 2018). This could enable attainment of higher conception rates than is currently possible using conventional oestrus detection methods (Grodkowski et al. 2018). However, for the successful implementation of accelerometer technology into current farming practices, automation of this information would be required (Labrecque & Rivest 2018). Accelerometer implementation would overcome the issue of skilled labour availability and ensure that animals displaying an oestrus event outside normal working hours still receive an insemination dose (Frost et al. 1997; Guo et al. 2006). This would enhance production efficiency by reducing the number of non-productive days incurred prior to mating (Abell 2011; Koketsu et al. 1997).

When using accelerometer technology, precision is a performance estimate that varies across the different behaviour types (Neilsen 2013). Quantification of simple behaviours such as grazing in cattle can produce results with a precision of 90-95% (Diosdado et al. 2015). As the behaviour becomes more complex, the precision reduces to ranges of 80-85% leading to more variable predictive power (Alvarenga et al. 2016; Dobos et al. 2016; Cornou et al. 2011). The current study identified precise classification within similar ranges for the complex behaviours of flank nosing and sow-to-sow mounting. However, urogenital sniffing had

considerably limited precision of 57.3% indicating it is a poor method for quantifying this behaviour. This indicates that the action involved in urogenital sniffing is not distinctive and there is an absence of a unique signal profile. Similarly, urogenital sniffing was the behaviour with the worst performance in terms of false negative and false positive outcomes, with 62.3% and 42.8% respectively, demonstrating that significant errors will occur if there are attempts to classify this behaviour. The weak response may be due to a lack of differentiation between the action involved in urogenital sniffing and the minute head adjustments that occur as part of normal daily activity. Additionally, the physical action of this behaviour is often lacking any detectable head motion as the process of sniffing does not require kinetic action (Mitcheson et al. 2008). In contrast, sow-to-sow mounting, which is a rapid, obvious and visually distinctive behaviour obtained the highest sensitivity and specificity resulting in high predictive power. The correct classification of mating behaviours relies on distinctive changes in the intensity of a behaviour, which will ensure sexual changes are distinguishable from other behaviours (Brown et al. 2013; Guo et al. 2006). This technique has been applied to copulation in sharks and similar intensity amplifications were observed (Whitney et al. 2010). As the observable behavioural differences between male-female copulation events and sow-to-sow mounting is indistinguishable, these results were expected.

The use of collars as a mounting system for sows was effective in recording acceleration signal profiles and did not cause any long-term changes in behaviour. However, three of the twelve observed behaviours monitored in both collared and non-collared sows were significantly different between the groups. A three-fold increase in the frequency of walking in collared sows suggests potential distress in sows, causing a disturbance to normal behaviour (Manning et al. 2017). This could be associated with difficulty in attaining a comfortable resting position when lying due to the inability to maintain a normal head position with the collar in place (Handcock et al. 2009). Alternatively, the presence of more instances of walking behaviour could be indicative of elevated adrenaline secretion. This can occur as a reaction to the stressful event of applying the collar, which can cause increased secretion of these hormones (Christensen & Galbo 1983). This increased level of activity transpired on the day after collar application but did not continue in the next day, suggesting acclimatisation to the presence of the collar was relatively quick. As a result, the collars should be attached to sows

a minimum of two days prior the onset of the behaviour of interest to ensure habituation to the presence of the collar.

Collared sows performed significantly less bouts of chewing than non-collared sows during the first day of observation post-collar attachment. The reason for this difference could be related to the adjusted levels of walking, possibly due to an increase in the level of adrenaline associated with the collar-attachment process (Christensen & Galbo 1983; Manning et al. 2017). Digestive function reduces when the level of adrenaline is elevated in preparation for the fight-or-flight response (McCorry 2007). Additionally, the collar was attached around the caudal side of the mandible, potentially disturbing the consumption of food and causing changes to the ability of the sow to swallow (Naito et al. 2010). Maintaining sufficient space between the surface of the skin and the collar is essential for preventing sow discomfort (He et al. 2008). However, as the difference in the number of feeding bouts was only observed on the first day of collar attachment, there was not an extended effect on animal welfare, feed intake or skin irritation from using this system.

Sows with collars were more likely to prefer lying on the left side of the body, with the right side of the body directed away from the surface of the ground. This supports the previously observed preference for sows to undertake left-side resting behaviours (Rolandsdotter et al. 2009). In the current study, all collars were orientated consistently but the metal buckle and extra length for securing the collar was present on the right side of the body, at the midpoint between the dorsal and ventral surfaces. Lying with this part of the collar positioned underneath the sow could cause discomfort and subsequent adjustment of body posture to lessen the effect. Alternatively, personal preference for lying on a specific side may be different for each sow (Gibbons et al. 2012). As there are no negative consequences for lying side preference, collar attachment would not present any sow welfare concerns (Thompson et al. 2016).

The addition of collars did not influence the expression of the remaining nine behaviours observed in this study. This is promising as it suggests nominal impacts on sow-to-sow interactions such as fighting and grooming or the manipulation of the collars, either by the individual to which the collar was attached or by other animals in the same pen. The differences

between sows with and without collars indicate that the collars altered behaviour minimally for less than 24 hours, so quantification of oestrus behaviours is possible without introducing negative stereotypical behaviours (Lawrence & Terlouw 1993). Previous studies identified that an acclimatisation period was not required for grazing cattle using collar-attached GNSS technology (Manning et al. 2017). However, the inquisitive and social nature of pigs, which causes more interactions between individual animals, appears to require a period of habituation (Wood-Gush & Vestergaard 1993).

The signal profiles developed in this study have been linked with specific oestrus behaviours. The development of algorithms that enable prediction of specific behaviours in real-time would revolutionise oestrus detection protocols (Alvarenga et al. 2016). Flank nosing and sow-to-sow mounting can be differentiated from other behaviours with accuracy rates of 78.6% and 89.5% respectively while increasing the cumulative overall activity level. Applying this knowledge can be as simple as providing individual stock-people with the signal profiles associated with flank nosing or sow-to-sow mounting to match with each new sow. Alternatively, development of a software program that communicates the unique signal profiles as a digital footprint indicative of oestrus to stock-people via a smart device could enable real-time, remote notification of the sows' reproductive status and instigate targeted inseminations for each sow (Borchers et al. 2016). By enabling stock-people to manage the insemination timing of individual animals with a high level of accuracy whilst reducing the need for labour input, the productivity and conception rates of the entire herd would be expected to increase compared with the current subjective oestrus detection methods. As well as improving the farrowing rates, other potential benefits include reduced wastage of semen, a decreased need for exogenous hormones, minimal labour input and fewer difficulties in skill attainment and retention of staff. Furthermore, the pork industry can present a viable smart technology that can demonstrate transparency in the production process from farm to plate.

5.5 Conclusions

Oestrus behaviours can be detected in sows through the analysis of acceleration signal profiles collected from collar-mounted sensors. The signal profiles of numerous behaviours including lying, standing, walking, foraging, flank nosing, sow-to-sow mounting, and urogenital sniffing were unique and identifiable using raw data extracted directly from the accelerometers, suggesting an effective measure for distinguishing and signalling these behaviours in commercial settings. The oestrus behaviours were detected with varying levels of precision. Flank-nosing and sow-to-sow mounting were accurately classified at rates of 78.6% and 89.5% respectively. Correct identification of urogenital sniffing was limited to 55.3% and hence, acceleration is less reliable for differentiation of this behaviour from other similar actions. The impact of the collar-mounted accelerometers on behaviour was minimal, but a habituation period of 24 h is recommended to ensure the sows' behaviour is indistinguishable from that of non-collared sows.

5.6 References

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Chapter 6

Multiple method oestrus detection

Chapter 6
Cumulative scoring system of multiple physiological and behavioural markers for improved prediction of insemination timing

6.1 Introduction

Oestrus detection in a commercial breeding farm involves regular observation of sexual behaviours indicative of oestrus (Knox 2011; Roca et al. 2006). Accurate identification of this sexual receptivity is essential to maximise conception rates, but it is labour intensive and often difficult to detect due to variation in oestrus length and the reliance on subjective detection methods (Behan & Watson 2005; Knox 2016; Pedersen 2007). It would benefit the entire industry to utilise multiple objective ovulation markers to improve the accuracy and precision of insemination timing.

Most oestrus detection protocols require the identification of visually observable behaviours in sows (Pedersen 2007). Checks for oestrus detection in sows are undertaken during the period following weaning to identify sows that are sexually receptive and are also implemented in the cases of failed conception (Ginther 2014) and late pregnancy loss (Bertoldo et al. 2009). While current oestrus detection protocols implemented in commercial sow production can enable conception rates of 80-90%, there are considerable labour outputs required to obtain these results (Roca et al. 2016). There is a need for skilled and experienced stock-people and regular, adequate time allocation as these behaviour observations can be subtle and are the only procedure used to determine the timing of insemination (Boessen et al. 2018; Friebel et al. 2016). Additionally, multiple consecutive inseminations are usually required at 24 h intervals as it is difficult to identify the exact moment of ovulation. The repeated inseminations ensure that conception occurs, but it results in substantial semen wastage and higher production costs as 2-3 doses are required (Weitze et al. 1994). Furthermore, some sows undergo an oestrus event that encompasses hormonal and physiological changes but are not accompanied by behaviour changes. These events are

referred to as “silent heats” as oestrus is not visually detectable and therefore these animals often miss an insemination (ten Napel et al. 1995). This leads to an increase in the number of non-productive days accrued for the sow, which represents reduced reproductive efficiency and causes a decrease in the overall herd conception rate (Knox et al. 2013). All these difficulties in detecting the optimum time for insemination indicates there is a need to develop an alternative approach for detecting oestrus (Glencorse et al. 2017).

Exploiting quantifiable measures of the physiological changes to the body leading up to oestrus will enable detection of the individuals who fail to display oestrus behaviours (Cornou 2006). These measures could also be used to identify sows that return to oestrus after a failed insemination (Vargas et al. 2009). Reducing the number of non-productive days for individual animals improves the functionality of the reproductive tract, enhances herd production efficiency and decreases the financial cost of production by $3-5 AUD per day (Abell 2011; Engblom et al. 2007).

Several quantifiable techniques for detecting the optimum timing of insemination have been examined in chapters 2-5 of this thesis. These chapters have identified several physiological changes that are associated with the immediate 24 h period prior to ovulation. The markers that indicate an oestrus event is impending are vaginal electrical resistance (ER), body temperature, quantified activity levels and cervical mucus traits including crystallisation, pH and sodium concentration. All of these physiological traits are useful indicators of oestrus independently (Escalante et al. 2013; Hidalgo et al. 2015; Luño et al. 2012; Luño et al. 2013). Combining multiple techniques to detect the optimum timing of insemination could enhance the conception rates obtainable. Additionally, it could provide the ability to identify ovulation with enough accuracy to warrant a reduction in the number of semen doses required for successful conception (Gillan et al. 2008).

Oestrus-based scoring systems have been used within cattle industries. These systems involve the construction of a scoring system for multiple oestrus markers (Van Eerdenburg et al. 1996). Points are assigned to each physiological change based on its usefulness as a predictor of insemination timing. A total numerical score is formed by adding the values obtained for each behavioural or physiological trait that represents a measure of observed

oestrus. To classify the presence of an oestrus state, the total score must exceed a threshold point level. This method may be particularly useful in oestrus detection on commercial farms where herd size, labour limitations and stock-person skill level can lead to difficulty in accurately determining the optimum insemination timing.

The aim of this study was to determine the predictive sensitivity of the combination of individual oestrus detection markers of vaginal electrical resistance, cervical mucus composition, body temperature and accelerometer-detected cumulative activity level. By determining the timing of physiological changes relative to behavioural oestrus onset and ovulation, the timing of insemination will be identifiable with higher precision and accuracy.

6.2 Materials and Methods

6.2.1. Experimental design

The methodology for this study was approved by The University of Sydney Animal Ethics Committee (Project number: 2013/5942). The data used for this study was mined from the experiments completed and recorded in Chapters 2-5. Each of the previous chapters examined individual physiological traits to detect any correlation with the point of ovulation. Any of these physiological markers that were found to be associated with ovulation were included in the current chapter. The data were recorded from Large White, Landrace and Duroc crossbred sows (n=84) housed at the University of Sydney Mayfarm piggery. Data were collected over a three-year period from November 2014 to February 2017 in a batch farrowing breeding system. Sows were housed on deep litter in groups during gestation and moved to farrowing crates prior to parturition. They were supplied with ad libitum water and a high energy ration during farrowing and lactation. Following weaning, the sows were moved to cement pens of approximately 14 m^2 in groups of 4-6 animals. Oestrus detection checks were performed from three days after weaning until two days after the last observed behavioural oestrus. The sows cycled naturally during the post-weaning period and were monitored twice daily (07:00 and 15:00) for signs of behavioural oestrus using a teaser boar and the back-pressure test. Individual sows were monitored to determine the presence of an oestrus event, and the associated oestrus markers were recorded. The data were collated at three time-points;

proestrus, the onset of behavioural oestrus and 24 hours prior to the point of predicted ovulation. Proestrus was defined as the time-point occurring 24 hours prior to the onset of behavioural oestrus. Behavioural oestrus was defined as an occurrence of the erect standing posture when pressure was applied to the back of the sow. The time-point for onset of behavioural oestrus was defined as the halfway point between the first instance of standing heat and last instance without standing heat. Predicted ovulation was termed as the point 30 hours prior to the peak progesterone concentration as determined from faecal samples (Snoj et al. 1998). The time-point of 24 hours prior to predicted ovulation was used as this provides sufficient time to enable identification of oestrus and allows an insemination to be performed before the end of the ovulation event. The physiological markers that were associated with detecting the ovulation time-point in chapters 2-5 are presented in Table 6.1. The methods used for collecting measurements for each marker are presented in the associated chapters.

6.2.2. Statistical analysis

Data was analysed in R (i386 v3.4.2, R Core Team (2017)) and a p-value of <0.05 was used to indicate a significant effect. The proportion of sows that demonstrated each physiological marker was calculated for the time points, with all time-points independent of each other. Individual two proportion z-tests were used to determine if the presence or absence of each desired marker differed between the time-points of behavioural oestrus onset and 24 hours pre-ovulation.

Subsequently, a scoring system was developed to enable the calculation of the likelihood of an ovulation or oestrus events. The scoring system was tested using a learning and validation dataset. In the learning dataset, a value was assigned to each physiological marker based on the proportion of sows that displayed the change during each time-point. A value was assigned to each marker based on the proportion of sows that demonstrated the specific physiological change in the learning dataset. This scoring system calculated the rate of certainty that ovulation was impending based on the accumulation of points from each marker. The proportions of the sample size that presented the desired change at the later time-point was divided by those at the preceding time-point to obtain the fold increase in sows demonstrating that trait. This value was used as a score to be allocated to each sow when the desired

physiological change occurred. A total score was calculated for each sow during the time-points. A threshold value that indicated that either oestrus or ovulation was occurring in a large proportion of sows was devised to mimic the approximate conception rates of 90% that occur in optimal commercial settings. The system was tested using a validation dataset.

Table 6.1 Criteria for the selection of desired changes in physiological oestrus markers occurring within the recording period as examined previously in chapters 2-5.

Marker	**Definition of the observed change that was associated with an oestrus or ovulation event**
Behaviour change	Immobile sexual posture of standing heat in the presence of a boar
Vaginal electrical resistance	A reduction in the recorded vaginal ER followed by a sharp increase
Mucus pH	Decrease in pH by 0.5
Mucus sodium concentration	Decrease in sodium concentration by 60 mmol/L
Mucus crystallisation pattern	A pattern that has an arrangement of short, linear shapes
Vulva temperature	Increase in temperature by 1.5°C

6.3 Results

Analysis of the faecal hormones indicated that the 84 sows included in the study underwent an oestrus event. The percentage of sows that underwent the desired change in the physiological markers across the varied time-points are displayed in Table 6.2. Throughout the recorded periods, standing heat was not detected in any of the sows classified as in proestrus. In contrast, all sows that were observed at the onset of behavioural oestrus displayed standing heat, as per the definition of this period. The 24-hour period prior to predicted ovulation identified 77 sows (91.7%) demonstrating standing heat. The markers that were most consistently associated with the 24 h pre-ovulation timepoint were standing heat and linear mucus pattern. The marker that was most closely associated with the onset of behavioural oestrus was the increased activity level as detected by accelerometer output. Vulva temperature had the lowest percentage of sows that displayed this physiological trait out of all the markers observed in the 24-hour period prior to ovulation.

Table 6.2 Percentage of sows (n=84) displaying the desired physiological marker recorded during each of the observed time periods. Mucus sodium concentration was tested in 19 animals only. Differing subscripts within a row indicate a statistically significant difference.

Physiological marker	Proestrus	Onset of behavioural oestrus	24 hours prior to ovulation
Presence of standing heat	0	100.0[a]	92.7[b]
Increase in vaginal ER	4.7	14.3[a]	63.1[b]
Low mucus pH	3.6	21.4[a]	64.3[b]
Linear mucus patterns	0	27.4[a]	81.0[b]
Decreased sodium concentration	5.3	10.5[a]	57.9[b]
Increased vulva temperature	19.1	26.1[a]	36.9[a]
Increased activity level	6.2	88.3[a]	78.4[a]

The fold increases associated with each desired change in physiological markers and the corresponding point allocation for each of these changes are presented in Table 6.3. These values indicate the number of sows that presented each physiological marker at the corresponding time-point. The total scores for all sows are presented for the interval from proestrus to the onset of behavioural oestrus and from the onset of behavioural oestrus to the 24-hour pre-ovulation time-point (Figure 6.1). These scores indicate the value assigned to each physiological marker and can be used to determine if the sow has reached a threshold value that indicates an oestrus event.

The interval from the onset of behavioural oestrus to the 24-hour pre-ovulation interval required a minimum score of 1 to ensure all observed sows were classified as sows in oestrus. Increasing the total score threshold to 4 points reduced the number of oestrus sows identified by 2% which achieves an oestrus detection rate of 98%. During the proestrus to behavioural oestrus onset interval, 35% of sows obtained a score of 0 for all physiological marker changes and only 27% of the sample size had high scores at this time-point.

Table 6.3 Fold increase in the number of sows (n = 84) displaying each physiological marker recorded between two time-based intervals. The fold increase also corresponds to the number of points allocated to each sow when the physiological markers were observed in the sow.

Physiological marker	**Interval from proestrus to oestrus onset**	**Interval from oestrus onset to 24 hrs pre-ovulation**
Increase in VER	3	4
Low mucus pH	6	3
Linear mucus patterns	27	3
Decreased sodium concentration	2	6
Increased vulva temperature	1	1
Increased activity level	14	0.8

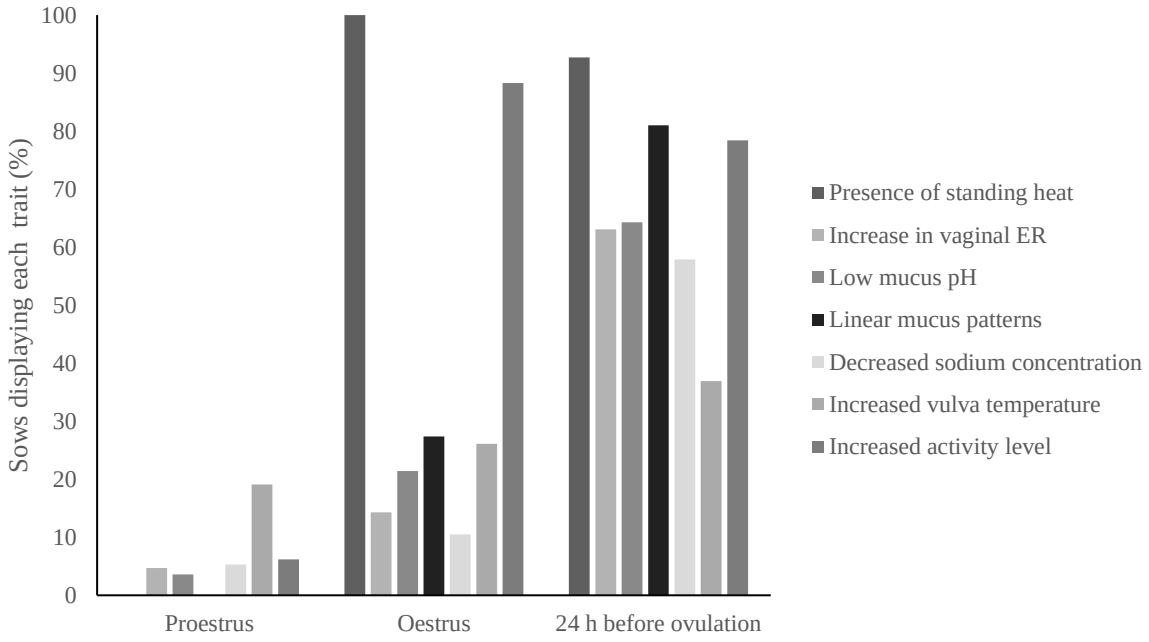

Figure 6.1 Percentage of sows displaying each of the physiological markers at each time-point during oestrus.

6.4 Discussion

This study verified that the use of multiple physiological markers combined into a cumulative scoring system gives an accurate indicator to determine optimal insemination timing. By waiting for multiple physiological changes to occur before deeming a sow to be in oestrus, identification of 98% of sows at the 24-hour pre-ovulation time-point was possible. This study was the first to contrive and examine if multiple markers could be used for oestrus detection in sows. A similar method has been used previously with success in cattle (Van Eerdenburg et al. 1996; Cowen et al. 1989). The focus of this study was to identify the physiological markers that are most useful for predicting the onset of oestrus and ovulation. The onset of oestrus is associated with an accelerometer-predicted increase in activity level while ovulation can be identified by the remaining physiological markers.

Accelerometer quantified activity was the only physiological marker that was useful for predicting the onset of behavioural oestrus. This accumulation of intense activity occurs during oestrus as the sow is attempting to court a mate (Pedersen 2007). This increased activity

level was detected in most of the sows during the pro-oestrus to oestrus interval, therefore making this marker the most suitable for detecting oestrus. The observation of accelerometer quantified activity level is associated with the behaviour traits that are used in conventional oestrus detection methods (Macmillan et al. 2020). This marker is useful as it is a quantification of the increase in activity associated with sexual behaviour (Johnson & Shade 2017). As a result, detection of a high activity level using this technology could replace the behaviour-based oestrus detection methods, such as the back-pressure test, that are currently used for predicting the optimum insemination timing (Arcidiacono et al. 2020; Wang et al. 2020). While all sows had the required presence of standing heat at the interval from proestrus to oestrus onset, there was an 8% reduction in the number of sows that presented this behaviour at the oestrus onset to 24-hour pre-ovulation marker. Therefore, ovulation cannot be identified accurately using accelerometer-based activity level alone because there was no observed increase in activity level at ovulation. This suggests that the accelerometer detected oestrus methods may result in false negative classification of sows resulting in them not being inseminated. However, this technology does have several benefits over the traditional back pressure test when detecting oestrus. Accelerometers are a technology that is capable of recording the activity of the sow at all times of the day (Lush et al. 2016; Nuijten et al. 2020). This allows for an accurate measurement of the total activity over the oestrus period instead of relying on a single, short observation period that is used within conventional oestrus detection (Neethirajan 2017). Additionally, it could facilitate decreased labour requirements by eliminating the need for frequent behaviour observation, and therefore removing the need for development of subjective skills in behaviour observation and analysis (Johnson & Shade 2017).

The onset of oestrus can be identified using activity level, but ovulation must be signalled using different physiological changes. Sodium concentration and vaginal ER underwent the most significant changes in the 24 h period prior to ovulation as shown by the largest fold increase. This outcome demonstrates that these two physiological changes were the most effective markers for ovulation.

Linear mucus crystallisation pattern was the most effective marker for prediction of ovulation. The composition of mucus in the reproductive tract involves an oestrus-specific

physiological change and therefore any substantial change could be indicative of cyclicity (Glencorse et al. 2017; Haynes 1971; Lee et al. 2013). Currently, cervical mucus in observed in commercial oestrus detection with stock-people monitoring any change in "stringiness" or viscosity (Zaaijer et al. 1993). This is a subjective, judgement-based observation with no quantification and therefore repeatability is low. Cervical mucus is not tested in all animals but is used as a complementary marker to apply alongside observation of standing heat when possible (Luño et al. 2012). In order to utilise our understanding of cervical mucus composition changes on pig farms, observation of mucus patterns under a microscope from the first instance of behavioural oestrus will assist prediction of ovulation timing. Detection of short, linear crystallisation patterns in air-dried mucus samples is a more time-consuming task and requires training. Nonetheless, use of this method would improve the detectability of the optimum timing of insemination and reduce the number of doses required for conception (Cortés et al. 2012; Glencorse et al. 2017; Luño et al. 2012).

The remaining physiological markers were not demonstrated consistently in all sows, which caused reduced precision in the detection of ovulation. However, refinement of the techniques involved in measuring these physiological changes could improve their functionality as a predictor of ovulation. The markers of pH and crystallisation are both simple methods for analysing the composition of cervical mucus and allowed provision of a score of 3 individually. Cervical mucus pH can be measured with commercially available indicator strips and is therefore a simple, effective method that enables a drop in the 24-hour pre-ovulation period to be reliably detected. Alternatively, sodium concentration could be measured using a near infrared spectroscopy (NIRS) device that allows frequent monitoring of mucus composition. This technology has been used for various molecules, but validation of sodium concentrations in mucus would be necessary before this technique can be recommended for commercial application (Kleinebecker et al. 2013).

Vulva temperature, a measure of inflammation of the reproductive tract has shown limited efficacy when used to detect the optimum timing for insemination previously (Luño et al. 2013; Talukder et al. 2014). This finding was supported in the current study. The primary weakness of temperature monitoring is the effect of ambient temperature, particularly in areas distal to the core of the body where environmental changes have significant effects on the body (Kessel

et al. 2010). Core body temperature has limited importance for temperature fluctuations in the reproductive tract as it is an isolated area that is directly influenced by oestrogen secretion (Czaja & Butera 1986; Loughmiller et al. 2001).

Additionally, ambient temperature interferes with temperature recordings at peripheral locations on the body, hence causing fluctuations that are not caused by oestrus (Kessel et al. 2010). Within the multiple marker scoring system assessed here, temperature was the least effective indicator of oestrus in sows. However, the limitation of this marker could be improved by using more advanced, continuous recording technologies (Wathes et al. 2008). Some of these technologies include continuous thermal imaging, which would enable tracking of temperature changes more frequently, hence leading to detection of changes in real-time (Zhang et al. 2019). Alternatively, internal thermometer implants enable constant monitoring of body temperature with the additional benefit of reducing the effect of environmental temperature on the external surfaces of the body (Green et al. 2005).

The accuracy of oestrus detection can be adjusted by using different threshold values for the scores defined in this study (Van Eerdenburg et al. 1996). The threshold value that allowed for the greatest accuracy with minimal false positive classifications was a score of four. This method required each sow to demonstrate several physiological changes with a total score greater than four in order to be classified to be in oestrus. This method allowed identification of ovulation with a higher level of accuracy than when using a single physiological marker. Obtaining a total score of 4, which detected 98% of oestrus sows, involved measurement of two physiological markers. By including observations of several physiological markers, the precision of oestrus detection protocols could be further improved by reducing the number of false negative classifications. In addition, by using objective and quantifiable physiological markers, the margin of error can be reduced. Alternatively, the detection rate of the current study was 100% when using a total threshold score of 1 at the interval between oestrus onset and the 24-hour pre-ovulation marker. This low score is attainable when observing a single marker. While 100% of sows in oestrus were detected, this often leads to an increase in the proportion of false positive oestrus events. This issue could present a risk that sows will be detected in oestrus too early for successful insemination.

Increasing the number of markers used and use of the threshold score devised here will prevent misclassification of oestrus status.

In the current study, the detection of the oestrus status of sows was possible when two daily observations of physiological measurements were recorded. This is a positive outcome for implementation of this protocol into commercial farms, as only minor additional labour is required. There is a substantial financial cost for labour associated with oestrus detection. In a breeding unit with 7,500 sows, oestrus detection requires a daily allocation of 1.5 full time equivalents as the time required for this task ranges from 5-15 min for each sow (Martel et al. 2008). As most large commercial farms rely on the use of daily observations of behaviour, a process that relies on significant time-consuming animal movement, the labour changes associated with application of this multiple-marker procedure is negligible (Van Eerdenburg et al. 1996). Despite these positive outcomes, the number of time-points for recording oestrus changes may have impeded the outcomes that are expected in this study. Twice daily observations occurred in this study, which has the potential to reduce the sensitivity of the outcomes. This is particularly relevant as the current study did not compare the total scores obtained from sows that did not undergo an oestrus event. Although more frequent monitoring of oestrus events would improve the sensitivity of this scoring system, commercial procedures on pig farms are limited to either a single or twice daily observation to detect oestrus and therefore the current approach would be suitable (De Rensis & Kirkwood 2016). While future studies could attempt to reduce the inter-observational length to increase precision, more frequent oestrus assessments are not realistic in a commercial setting.

The proestrus to oestrus onset interval is not an effective time-point for identifying oestrus changes which was demonstrated by 35% of sows displaying no identifiable physiological changes. As a result, marker changes in this interval are not capable of reliably detecting the optimum mating timing. This supports previous findings that some sows do not present obvious visual changes to enable identification of the optimum insemination timing (Merks et al. 2000). Changes in physiological markers cannot be reliably used to detect the change from proestrus to the onset of behavioural oestrus and therefore this marker interval is not appropriate for detecting insemination timing.

The reproductive status of individual sows who do not display obvious behavioural changes at the onset of oestrus has been examined previously (Špinka 2009). The current study identified a disconnect between the onset of oestrus and the timing of changes to physiological markers, suggesting that the secretion of oestrogen may be a factor in these transformations. Oestrogen secretion increases progressively through the oestrus period, corresponding with the growth of follicles in the ovary (Soede et al. 2011). The effect of oestrogen appears to have had the same progressive effect throughout oestrus in the current study, suggesting that the physiological changes to the reproductive tract and associated fluids occurs as the concentration builds within the sows' circulatory system (Soede et al. 1994). This trend suggests a need to transition the focus of oestrus detection from the current approach of identifying oestrus onset to concentrating on identifying the physiological effects of oestrogen on the body, when maximal oestrogen levels are circulating. This cause-and-effect understanding of reproductive processes is often lacking in commercial farms but can be overcome by using the markers examined here to identify the true reproductive status of the sow.

An alternative scoring system was used by Hall et al. (1959) in a study that used an intensity-based scoring system for each oestrus marker. This is beneficial as it demonstrates the amplification of the marker which is associated strongly with the oestrus period. However, this tactic is limited by subjectivity as the stock-person is required to make decisions based on an unquantifiable visual scale. As this type of scoring system is subjective and non-quantifiable, it would be expected to produce similar results to existing oestrus detection procedures used on commercial farms and therefore would not provide an improvement on current methods.

Previous studies have required observation and identification of more markers to reach the higher threshold levels necessary to identify the oestrus status (Van Eerenburg et al. 1996). Additionally, these scoring systems rely on identifying a change in the behaviour, which is a difficult task for unexperienced stock-people and relies on the ability to recall behaviours that were occurring at previous inspections. This study indicates the value of using physiological markers, as behaviour-based changes are flawed by the associated subjectivity. Furthermore, the scoring system described here is easy for inexperienced or untrained staff to use. All

physiological markers, except for vulva temperature had a score of 3 or more allocated for identification of this change. This would enable individual farms or staff to select their preferred marker, hence allowing choice and responsibility. The markers used are quantifiable and objective, hence removing the variation inherent with the current behaviour-based observation methods used in commercial farms.

6.5 Conclusions

The onset of oestrus can be detected by the presence of elevated activity level as predicted by accelerometer output. The detection of oestrus at the 24h pre-ovulation timepoint is most accurate when a linear cervical mucus crystallisation pattern is observed. Using multiple markers to predict the optimum timing for insemination will enable greater accuracy. Multiple marker oestrus detection provided a 98% detection rate of sows in oestrus at the interval between the onset of behavioural oestrus and the 24-hour pre-ovulation marker. A minimum of one physiological marker is required to enable detection of 98% of sows in oestrus. To ensure accurate and precise detection of the optimum timing for insemination, stock-people should identify a sharp increase in vaginal ER, a reduction in mucus pH and sodium concentration, the presence of linear crystallisation fragments, elevated vulva temperature and accelerometer-quantified activity level. A combination of two or more of these physiological markers will improve the precision of current oestrus monitoring leading to maximised conception rates using a single insemination dose.

6.6 References

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Chapter 7

General Discussion

Chapter 7

General Discussion and Conclusions

7.1 Introduction

This study investigated innovative markers for determining the optimum timing of insemination and identified an alternative oestrus detection protocol that is objective, reliable and repeatable. An alternative, optimised approach to traditional oestrus detection was required to achieve maximal productivity and profitability while ensuring that each animal receives adequate attention and care. As the demand for food production increases, animal industries must improve their operations to ensure that the supply of meat is sufficient. Intensive production of animals such as pigs is more sustainable than extensive farming of cattle as it enables higher stocking densities and therefore more efficient use of the available land. Larger herd sizes enable production of a greater volume of meat, but intensive farms can be limited by labour availability.

One area that requires improvement is sow breeding management, because reproductive success is highly variable within and between farms. A typical artificial insemination (AI) breeding protocol involves two or more doses of semen, 12 h apart, with a dose rate of 2.5-3 x 10^9 spermatozoa deposited into the cervix following the first observed instance of behavioural oestrus expression upon exposure to a boar (Broekhuijse et al. 2015; Soede et al. 1995). Oestrus detection is a fundamental process that enables identification of sows that are sexually receptive. The myriad of studies into ideal timing of AI agree that accurate oestrus detection is crucial, but this is a major limitation in a commercial setting, hence the use of multiple insemination doses to cover for the inaccuracies of current oestrus detection methods (Knox 2016). In part, this is due to the subjective nature of the oestrus detection process, variability in oestrus length and behaviour expression between females, most notably in gilts, and an inability to detect ovulation precisely in real-time (Halachmi et al. 2019). The process also requires experienced stock-people and labour input in addition to staff training to ensure competency (King & Macpherson 1965). Staff turnover is high due to the poor

perception of career progression and the requirement for regional relocation. These factors can cause poor oestrus detection rates and variable, inconsistent conception rates (Koketsu & Iida 2017).

A unique approach to overcoming the limitations of single AI programs is to implement detection protocols which focus on identifying objectively measurable physiological changes associated specifically with ovulation and the immediate 24 h period prior to it rather than focusing on observational changes across the entire oestrus period (Roca et al. 2006). A more precise and objective oestrus detection tool will enable producers to achieve equivalent or improved conception and farrowing rates, reduce semen wastage, eliminate the need for exogenous hormones for optimizing insemination, overcome difficulties in skill attainment and retain farm staff, all of which lead to increased herd productivity and breeding efficiency. The potential of a variety of these methods was investigated to determine the effectiveness for translation to industry. In chapters 2 to 5, four oestrus-associated physiological mechanisms were quantified to assess if these changes could be used as an alternative marker for determining the optimal insemination timing. To the authors knowledge, none of these methods have been incorporated into an objective, real-time protocol suitable for replacing current oestrus detection procedures in a commercial setting.

7.2 Beneficial oestrus and ovulation markers

Any markers that undergo changes in the 24 hours prior to ovulation will enable inseminations to be conducted 12 and 24 hours after the observed change, hence facilitating timely deposition of viable spermatozoa. The markers that underwent a detectable change in this part of the oestrus cycle will be discussed here.

Tri-axial accelerometers have been implemented to detect and quantify movement in order to enable prediction of the behavioural changes that are typically used to classify a sows' oestrus status (Alvarenga et al. 2016; Halachmi et al. 2019; Ramonet & Bertin 2018). Lying, standing, walking, foraging, flank-nosing and sow-to-sow mounting each have distinctive signal profiles that are easily distinguishable. Flank-nosing and sow-to-sow mounting were

successfully differentiated from the other behaviours detected with accuracy rates of 78.6% and 89.5% respectively and were only identified in sows who displayed an oestrus event.

Prior to ovulation, the reproductive tract undergoes changes in preparation for deposition of spermatozoa. Several visible and molecular changes were observed throughout this study, with each change representing a potential marker for determining the optimum timing of insemination. A temperature increase was observed on the external surface of the vulva during early oestrus indicating that temperature can be used to identify an oestrus event. There was an increase in the external vulva temperature by 1.4°C that occurred 24 h prior to ovulation. The timing of this change suggests that increased ovarian hormone secretion leads to vasodilation in the vulva which causes an increased supply of blood to the reproductive tract and an associated increase in heat production (de Ruediger et al. 2018; Scolari et al. 2011).

The internal epithelial lining of the reproductive tract underwent several additional changes. Previous studies documented that cervical mucus undergoes gross composition changes during oestrus, under the influence of increased oestrogen concentration (Luño et al. 2012). There is an increased volume of cervical mucus at this time, along with an increased concentration of components such as sodium chloride, phosphates, bicarbonates and mucins with the latter producing the viscoelastic properties (Luño et al. 2012). The arrangement of mucus changed from large irregular shapes to short, linear streaks 24 h prior to ovulation. Additionally, the ER of vaginal mucus decreased to basal levels during early oestrus followed by an increase to a peak in the 24-hour period prior to ovulation, indicating that a sharp rise in vaginal ER is an observable flag for conducting insemination. Furthermore, a decline in cervical mucus pH 24 h prior to ovulation was observed, indicating that an acidic environment causes decreased secretion of mucosal fluid, which allows for reduced viscoelasticity to facilitate transit of spermatozoa (Espinosa et al. 2002; Mather & Day 1977). The change in acidity within the reproductive tract is required to prepare for the arrival and passage of spermatozoa to the oviduct for fertilisation (Rodriguez-Martinez 2007).

While previous studies have identified that vaginal ER is not sufficiently accurate for the prediction of ovulation (Hidalgo et al. 2015), the current study determined that these changes were correlated with the time-point 24 h prior to ovulation. This study demonstrated

high conception rates when AI was performed with a double insemination using liquid-stored semen following the first sharp rise in vaginal ER. A single dose insemination program could be commercially viable if semen is deposited 12 hours after the increase in vaginal ER, hence facilitating a reduction in semen wastage and reducing the labour requirement by half. While conception rates were not commercially acceptable when frozen-thawed spermatozoa were inseminated 12 and 24 h after the increase in vaginal ER, further refinement of the timing of AI may improve the rates of successful fertilisation with such compromised spermatozoa.

The observation of these alternative markers informs refinement of a practical oestrus detection method for large-scale production units. Inseminations that occur after identification of the required mucus pattern will allow for consistent inseminations to be conducted at a fixed time relative to ovulation without the need for exogenous hormones. Further investigations should focus on conducting inseminations immediately after these observed changes to the reproductive tract. It requires a small investment in stock-person training to provide individuals with objective, problem-solving skills. Such a technique for detecting oestrus can overcome issues with inconsistent conception rates by maintaining consistency in staff training that will allow assessment of the oestrus status of the sow.

7.3 Potential adoption of alternative oestrus monitoring tools and techniques

There are several commercially available tools that can be applied to oestrus monitoring. The probes available for ER monitoring use a simple design with uncomplicated instructions, and therefore staff training is easy. The major requirement for accurate ER monitoring is implementation of consistent probe contact. The use of precise ER probes would reduce the labour required to detect oestrus compared with conventional behavioural observation.

Infrared and thermal thermometers were compared to determine temperature thresholds or time-dependent fluctuations that correlate with ovulation (Luño et al. 2013). There was substantially more variation in temperatures recorded at all locations when using a simple, handheld infrared gun. The more sophisticated thermal imaging software facilitated more

consistent and less variable results than the infrared gun (Warriss et al. 2006). Investment in advanced infrared thermal imaging technology is more beneficial than attempting to conduct temperature monitoring with simple handheld devices (Zhang et al. 2019).

There is a potential for development of a device that can quantify the changes in cervical mucus crystallisation to remove the need for stock-people to analyse the patterns. A simple indicator test strip could be used to measure the concentration of specific ions in the cervical mucus that cause the changes in crystallisation pattern. This would reduce the requirement for decision making on when to conduct inseminations and provide a further reduction in labour. The current study identified an increase in sodium concentrations in the mucus present in the 24 h period prior to ovulation. Further investigation into the molecular composition of mucus prior to ovulation can be conducted using near infrared spectroscopy (NIRS), which is an absorbance-based assessment tool. The use of NIRS to determine the composition of biofluids such as mucus has become increasingly attractive as the technology becomes more user-friendly and portable (Kleinebecker et al. 2013). This can be implemented as a commercially available pen-side test for use in pigs across Australia and internationally. The success of this innovative application depends upon a correlation between NIR-detected mucus composition and ovulation.

A unique crystallisation classification method for describing the distinctive microscopic patterns in air-dried mucus samples was tested. The classification guidelines for cervical mucus crystallisation patterns must be transferable and repeatable for multiple, scientifically trained and industry employed participants. Agricultural workers were able to correctly classify the category associated with the 24-hour pre-ovulation time-point. Moreover, respondents preferred visual classification guidelines over descriptive guidelines with the former leading to higher accuracy and precision in detection. These outcomes suggest that implementation of cervical mucus analyses would be an effective, subjective and adoptable tool to enhance or replace the current oestrus detection procedures.

Accelerometer signal profiles are valuable quantifications of behaviours in the raw format, but the value of this information is enhanced if it is processed and available in a real-time, automated system (Ramonet & Bertin 2018). An example of this technology is provided

by BioPac, which produces an accelerometer system that was originally designed for monitoring athlete motion. The accelerometer is accompanied by a system that allows visualisation of the signal profiles on a handheld device in real-time (Halachmi et al. 2019). Stock-people could use this device in the pen during oestrus detection to assess the oestrus status of individual animals and decide if the sow has presented the signal profiles that represent an oestrus event. This method is favourable as it requires minimal equipment training and does not require stock-people to be experienced. Such a system would revolutionise commercial oestrus detection by enabling inseminations to be consistently identified. Alternatively, production of a national livestock identification system (NLIS) or transponder linked device that can record acceleration patterns and return an alert to an external smart device would allow stock-people to make decisions in real-time (Alvarenga et al. 2016). This concept is currently available in various commercial products such as CowManager within the dairy and beef industries (Hodson & Timms 2017). This has wide potential for use in pigs not only in reproductive management but also for health, disease and parturition detection. Acceleration-based behaviour quantification has allowed for identification of the oestrus period in sows, but it has unlimited applications across the pig industry. Initial refinement of these technologies is an intensive task that requires significant investment in scientific research and cooperation from commercial producers. However, the difficulty in this research area comes from committing to complete this sizable undertaking. Once the technological framework has been developed, it will be easy to apply these frameworks to develop predictive algorithms for monitoring other areas within the pig production chain. Some potential areas of interest include, but are not limited to monitoring feed consumption to identify sows that are undergoing changes in body condition (Farooq & Sazonov 2018), identifying reduced activity levels that indicate development of illnesses or conditions that require treatment (Barker et al. 2018; Koltes et al. 2018; Martínez-Avilés et al. 2017), detecting aggression in group housing and during mixing (Lancaster 2018; Ramonet & Bertin 2018) and recognising distress in animals that could lead to welfare concerns (Cui et al. 2019).

The method used to attach accelerometers is crucial and a particular challenge in pigs. Therefore, this study assessed if collar-based accelerometer-mounting would cause a change in behavioural expression or lead to distress. There were minimal impacts to behaviour

expression from day two following attachment suggesting that a two-day habituation period for collar attachment prior to examination of desired behaviours is recommended. Although this study enabled successful behaviour quantification using a collar-based accelerometer, this method would be difficult to implement on a commercial farm. An alternative attachment method involves the use of an ear tag. Many large pig farms utilise automated feeding systems that rely on radiofrequency identification (RFID) transponders, which are attached to the animal on an ear tag. Ear tags have less impact on animal behaviour than collar-based attachment, but further studies are needed to determine whether the ear is a suitable location for identifying different sow behaviours.

7.4 Poor alternative markers

The vestibule undergoes changes in conductivity that mimic those of the vagina. Using this measurement for timing inseminations would be expected to result in conception rates comparable with traditional behavioural oestrus detection. The current study identified that vestibular ER undergoes a distinctive low followed by an increase 12 hours prior to ovulation and therefore may not provide sufficient notice to allow for inseminations to be conducted. Therefore, vestibular ER was regarded ineffective as a predictive marker of ovulation. In addition, the variation in vestibular ER readings was higher than that of vaginal ER, potentially due to inconsistent probe positioning and the dynamic, heterogenous mucus composition.

The prediction of ovulation via body temperature monitoring was the method with the least potential for commercial adoption of those investigated in this thesis. The changes to body temperature were highly variable across sows and were influenced by the ambient temperature. Thermal imaging technology could incorporate ambient temperature calculations to quantify and account for the fluctuations in skin temperature that occur as a result of air convection (Warriss et al. 2006). If this refinement is possible, temperature assessment would become less variable and present a more viable option for determining the optimal insemination timing. There have been contradictory outcomes observed in this area of research with some results demonstrating conflicting temperature changes in the peri-ovulatory period. In the interval between proestrus and oestrus, an increase in the temperature of the vulva has been observed, indicating a possible early marker for oestrus (Bressers et al. 1994; Sykes et al. 2012). Contrary

to the finding of the current study, some studies did not observe a change in the temperature of the vulva or any other body location during oestrus (Soede et al. 1997). This issue may be due to the frequency of data recording. Temperature fluctuations occur constantly and therefore, to detect any significant change, several observations are required. Both the current study and previous studies have relied on 2-4 daily observations using hand-held devices rather than continuous monitoring. Continuous monitoring of temperature could be conducted using implanted, surface attached or video monitoring thermometers (Green et al. 2008; Zhang et al. 2019) and accurately measure temperature at higher sampling frequencies (Green et al. 2005).

Accelerometer detected urogenital sniffing behaviours did not present a distinctive signal profile that could be differentiated from other behaviours. The action of urogenital sniffing has poor predictive power for identifying the oestrus period in sows due to potential misclassification. The recurrent, vertical head movement involved in this behaviour is very similar to the motion associated with rooting and foraging and therefore it is not distinctive enough to separate the two actions using signal profiles. These outcomes indicate that accelerometer technology can only be used for identification of conspicuous, distinct and unique behaviours.

The results of this experiment could be enhanced by calculating a numerical value for the level of activity demonstrated during the oestrus period. A cumulative activity index to measure the intensity of movement has been used in cattle where these animals were found to have a marked elevation in overall activity during oestrus (Arcidiacono et al. 2018). The intensity during oestrus was higher than during proestrus, suggesting that the greater movement was a result of proceptivity (Pedersen 2007). If this method could be applied to produce a numerical value for the activity levels of sows during oestrus, it would further advance the current oestrus detection methods.

7.5 Multiple marker oestrus detection

As discussed above, Chapters 2-5 demonstrated that individual physiological markers can be used for the detection of the optimum time for insemination in sows. Subsequently, Chapter 6 investigated the use of multiple-marker oestrus detection to determine if ovulation

can be identified with greater accuracy and precision. The markers that were incorporated into the multiple-marker evaluation were vaginal ER, vulva temperature, activity level and the cervical mucus characteristics of pH, sodium concentration and crystallisation pattern. The aim of the study was to determine the combination of physiological markers that allow more precise identification of the optimum timing of insemination.

The only marker capable of detecting the onset of behavioural oestrus with high accuracy was accelerometer-quantified activity level. There was an 82% increase in activity level from proestrus to oestrus, potentially caused by an increase in the frequency of sexual behaviours. This clustering of activity occurs in other species during oestrus as females attempt to identify and court a possible mate (Aungier et al. 2015). Increased activity levels can be used to identify an oestrus event but cannot enable precise detection of the specific moment of ovulation as there is no observed increased in activity level at ovulation. Accelerometer-detected activity level could replace existing oestrus detection methods as it allows for quantification of sexual behaviour with accuracy and repeatability. Not only will this allow for a reduction in labour requirements by removing the need to observe the behaviour changes of sows each day, it also reduces staff skill development and experience requirements. Additionally, accelerometers produce more comprehensive assessments of behaviour as they record activity level for 24 hours of the day while stock-people are only able to observe behaviour changes in each animal over a 15-20 min period.

While activity level can be used to identify the onset of oestrus, other markers would be more beneficial for signalling ovulation. There was a 6-fold increase in the number of sows demonstrating an increase in mucosal sodium concentration just prior to ovulation and a 4-fold increase in the number displaying a sharp increase in vaginal ER. To utilise this knowledge on pig farms, a commercially viable method of measuring the concentration of sodium within cervical mucus needs to be devised. A near infrared spectroscopy (NIRS) device has been proven to be suitable for measuring the composition of liquids with the added benefit of rapid analysis, hence allowing results to be obtained in real-time (Kleinebecker et al. 2013). This technology has been used to monitor the reproductive status of giant pandas via urine, detecting oestrus in cattle through assessment of milk content and quantifying blood hormone concentrations in mares (Agcanas et al. 2017; Kinoshita et al. 2010; Takemura et al. 2015).

Testing and validation of NIRS technology is required to determine if rapid, real-time quantification of the sodium concentration in cervical mucus can be useful for oestrus monitoring and classification of the reproductive status of sows. To assess the practicality and labour requirement of a pen-side NIR oestrus detection device, these markers must be evaluated in a large commercial setting. In addition to being a highly accurate predictor of ovulation, any alternative oestrus method must have a reduced labour requirement and be easy to use in order to justify changes to farm protocols.

Using mucus pH, mucus crystallisation patterns, vulva temperature and accelerometer-detected activity level as indicators for ovulation may result in misclassification of the animal's status. While these markers lack precision for predicting the optimum insemination timing, further refinement of these techniques could improve their effectiveness. Temperature monitoring has demonstrated variable outcomes due to the rapid and frequent fluctuations that occur naturally and the influence of ambient temperatures. These outcomes can be refined by using a thermometer capable of continuous recording (Zhang et al. 2019). A tympanic thermometer mounted on a permanent ear tag could allow for continuous temperature recording and more precise detection of any significant change that signals the optimum insemination timing. These thermometers can be aligned with technology that records and processes the data using pre-programmed algorithms and sends alerts to an external smart device allowing stock-people to make decisions in real-time (Hillman et al. 2009). This application of thermography warrants attention as there is potential for exploitation of data for both oestrus detection as well as for monitoring other temperature-associated changes to the body such as illness and parturition.

7.6 Overall remarks

Despite the pig industry being one of the most data-rich animal industries with access to ample records and figures from every aspect of pork production, the information mined from these systems is minimal. Extracting valuable information that can be used to initiate more responsible, targeted management decisions will enhance the reproductive knowledge of stock-people and promote more accurate oestrus detection and effective insemination protocols through data-driven decision-making.

A weakness of using any technology to detect the optimal insemination timing is the requirement for training of stock-people prior to implementation. While this is a challenge for effective commercialisation, training staff to use objective techniques will result in more consistent decision making and a reduction in biased interpretation, compared with subjective methods, which rely on experience and practice. Uncomplicated methods such as the use of thermometers to measure vulva temperature and indicator strips to measure mucosal pH or sodium concentration do not require extensive training (Mather & Day 1977). Another issue with unskilled stock-people is the high staff turnover, forcing management to frequently train replacement staff. Use of technology that allows for impartial, objective decision making after a small investment in staff training will ensure insemination occurs close to ovulation, leading to maximised conception rates. It would also reduce the amount of labour allocated to breeding and insemination management from approximately 15 minutes per animal using currently utilised oestrus detection methods to 1-2 minutes per sow.

The marker most capable of accurately and precisely detecting ovulation was cervical mucus sodium concentration. A significant reduction in sodium was observed in cervical mucus 24 h prior to ovulation. Future studies should determine if inseminations conducted when this physiological marker occurs can result in conception rates comparable or even superior to those obtainable using behaviour-based oestrus detection methods. Additionally, this marker presents the greatest opportunity for testing if a single insemination can ensure effective conception. This would reduce the labour requirement of oestrus monitoring and potentially halve the cost of semen used in commercial production. In addition, to overcome the need for boars in commercial production, a more focused examination of these physiological markers during the full length of ovulation is required to determine if these methods can be used without any boar presence. By removing the need to utilise subjective behaviour analyses for oestrus detection, these novel, objective and quantifiable methods can be used effectively as an alternative and innovative approach to oestrus monitoring.

By combining a thorough understanding of the oestrus status of the animal with technology that can produce real-time decision-making capabilities, commercial producers will be able to improve the efficiency of reproductive management. Real-time notification of animal needs will enable more targeted decision-making and thereby improve production outcomes,

enhance animal welfare, reduce wastage of semen and the labour associated with AI and maximise profitability (Halachmi et al. 2019). Furthermore, these technologies have developed viable smart opportunities that provide transparency in the production process from farm to plate for consumers.

7.7 Conclusions

The current work identified several physiological markers that can be used to identify oestrus and the optimum insemination timing more precisely. Oestrus can be identified when elevated activity levels are recorded using collar-mounted accelerometers. The unique signal profiles for the distinctive sexual behaviours of flank nosing and sow-to-sow mounting can also predict oestrus with high accuracy. To ensure high conception rates, insemination should be conducted when ovulation markers are present. The recommended oestrus detection protocol that was established in this study indicates that inseminations should be conducted when a sharp increase in vaginal ER levels, a reduction in mucus pH and sodium concentration, the presence of linear crystallisation fragments and elevated vulva temperature are observed. If these physiological changes are observed, the sow can be classified as sexually receptive and an insemination should be conducted immediately. These markers all occur 24 h prior to ovulation, which provides sufficient lead-time to ensure inseminations can occur at an appropriate time for successful conception.

This is the first study to assess the effectiveness of multiple physiological markers when identifying the optimal timing of insemination. Using multiple methods increases the predictive power compared with using any of the methods individually. Ovulation can be predicted with precision by using accelerometers to detect the oestrus period and then by monitoring changes in cervical mucus sodium concentration or vaginal ER. Separately, these markers have great potential to increase the accuracy and efficiency of oestrus detection in sows and gilts. Combined, they present an innovative opportunity to transform oestrus detection into a productive, precise and efficient process that reduces labour and increases profitability in the pig industry.

7.8 References

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