Review: Understanding the Causes of Variation in Reproductive Wastage Among Bulls

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Short title: Variation in bull fertility

Abstract:

The ability to predict the fertility of bulls before semen is released into the field has been a long term objective of the animal breeding industry. However, the recent shift in the dairy industry towards the intensive use of young genomically selected bulls has increased its urgency. Such bulls, which are often in the highest demand, are frequently only used intensively for one season and consequently there is limited time to track their field fertility. A more pressing issue is that they produce fewer sperm per ejaculate than mature bulls and therefore there is a need to reduce the sperm number per straw to the minimum required without a concomitant reduction in fertility. However, as individual bulls vary in the minimum number of sperm required to achieve

their maximum fertility, this cannot be currently achieved without extensive field-testing. While an *in vitro* semen quality test, or combination of tests, which can accurately and consistently determine a bull's fertility and the optimum sperm number required represent the 'holy grail' in terms of semen assessment, this has not been achieved to date. Understanding the underlying causes of variation in bull fertility is a key prerequisite to achieving this goal. In this review, we consider the reliability of sire conception rate estimates and then consider where along the pregnancy establishment axis the variation in reproductive loss between bulls occurs. We discuss the etiology of these deficiencies in sperm function and propose avenues for future investigation.

Keywords:

Sperm, semen, bovine, prediction, fertility

Implications

A substantial number of bulls whose semen passes the post-thaw quality control checks in artificial insemination centres have reduced fertility in the field. While this is undoubtedly multifactorial, the purpose of this review is to attempt to identify where in the sequence of events, sperm from low fertility bulls compromise the establishment of pregnancy. Understanding this will aid in the development of improved strategies for the early detection of bull subfertility and/or its amelioration.

Introduction

Animal breeding centres have for years relied upon classical microscopy-based techniques to assess sperm motility (total and progressive) and morphological

parameters as part of their quality control programmes. However, work by our group and others have demonstrated that bulls whose semen passes these minimum post-thaw quality control checks at an artificial insemination (AI) centre can still vary in their field fertility. Traditional progeny testing schemes allowed semen from individual bulls to be released over a prolonged period and once non-return rate data became available, semen from subfertile bulls could be taken off the market. To protect against the risk of reduced fertility, AI companies typically utilise excessive sperm numbers in each straw (15-20 million). A number of studies with frozen-thawed conventional semen has revealed that most Holstein sires used in AI achieve their individual maximum pregnancy rate value at 2.5 million to 5.0 million total sperm per dose, with a range from 0.5 million to 12 million sperm per dose (Den Daas *et al.*, 1998). While the blanket approach of increasing the sperm number in all bulls guards against individual bulls with compensable sperm defects, this approach limits the number of straws that can be processed per ejaculate, thereby limiting supply of their semen.

With the advent of genomic selection, semen is now being collected from bulls at a younger age and these elite bulls are typically only used intensively for one season as they are then surpassed by the next generation of genetically superior bulls. This intensive use and high rate of AI sire turnover leaves insufficient time to adequately assess the fertility status of a bull prior to wide scale use of his semen in the field, especially in seasonal grass-based production systems, such as those operated in Ireland and New Zealand. In these pasture-based systems, the breeding season is condensed into approximately 3 months so as to calve cows compactly at the start of the grass-growing season. These young bulls also produce fewer sperm per ejaculate and therefore the luxury of putting excessive numbers of sperm in a semen straw for bulls where demand for semen far exceeds supply is costly for the AI centre and limits

75 farmer access to such elite bulls. Therefore, a reliable *in vitro* test, or a combination of
76 tests, which could accurately predict the outcome of an insemination would facilitate
77 the identification of subfertile bulls prior to their widespread use in the field and the
78 more efficient use of the semen of high fertility bulls through the reduction of sperm
79 number per straw.

80 There are numerous recent studies and comprehensive reviews on the prediction of
81 bull fertility (Sellem *et al.*, 2015, Utt, 2016, Abdollahi-Arpanahi *et al.*, 2017) and we do
82 not propose to replicate these here. Instead, this review will focus on the caveats
83 surrounding sire fertility estimates and on the specific reasons why bulls with
84 apparently normal semen vary in their fertility. We assess the usefulness of *in vitro*
85 assessments to mimic the *in vivo* events leading up to the establishment of a
86 pregnancy. Finally, we propose likely avenues for fruitful future investigation.

87

88 **Sire fertility estimates; Establishing a reliable phenotype**

89 Many studies that use *in vitro* approaches to investigate bull fertility fail to understand
90 the limitations of even the best designed sire fertility estimates and thus many studies
91 are flawed from the start due to an unreliable fertility phenotype. To accurately rank
92 sires, a detailed understanding of factors affecting the models are required. Most AI
93 centres worldwide track bull fertility using either non-return rates or more accurate (and
94 complex) adjusted sire conception rate (SCR) models that account for environmental
95 factors (herd, technician, month of insemination, age of cow, cow genotype, days in
96 milk, milk production, etc.) and express a bull's fertility relative to a population mean
97 of 0%. A detailed review by Amann and DeJarnette (2012) demonstrated that the
98 fertility of 90% of the bulls marketed is within ±3 percentage points of the mean of the

bull population. This is consistent with our preliminary data on Irish AI bulls. On a population basis, Amann and DeJarnette (2012) concluded that AI companies will never be able to measure "fertility" more precisely than ±3 percentage units from the population mean because of the difficulty in controlling many factors including: binomial variation, herd environment, measurement errors, and bias in semen use. Another key attribute in understanding 'Sire Fertility' is the number of inseminations required per sire to confidently rank them on their fertility. The same study illustrated that to confidently (2 tailed test, P = 0.05, 80% power) differentiate sires ±4% from the average of the bull population each sire must have a minimum of 1000 inseminations. With just 300 inseminations, as is often the case in studies attempting to predict the fertility of an individual ejaculate, it is only possible to confidently differentiate sires ±7% from the average. Thus, failure to recognise limitations in any estimate of potential fertility leads to over interpretation of small differences among sires in apparent fertility.

Do AI bulls rank the same when used under different conditions?

While the timing of insemination relative to onset of oestrus does not influence the fertility of above average fertility sires, a significant drop in fertility was reported when semen from below average sires was inseminated in early and mid-oestrus (Macmillan and Curnow, 1977), suggesting differences in the fertile lifespan of sperm in the female tract. Despite this fact, once a day AI is now widely used with similar fertility achieved to when twice a day AI is performed, irrespective of whether fresh or frozen-thawed semen is used (Xu, 2017).

Optimum fertility can be achieved with a much lower sperm number when fresh (liquid) rather than frozen-thawed semen is used (2 to 5 million versus 15 to 20 million sperm, respectively; Murphy *et al.*, 2017), maximising the utilisation of genetically superior sires. The higher sperm numbers in cryopreserved semen compensates for the damage during the freeze-thaw process compared to fresh semen and, on average, the same level of fertility is achieved with both types of semen (Murphy *et al.*, 2015). In a dataset analysed by our group, bulls which have low fertility with frozen-thawed semen tend to have low fertility with fresh semen although there are some exceptions (Figure 1). Of the 16 bulls used across 66,252 inseminations, 9 bulls varied substantially in the fertility achieved between fresh and frozen-thawed semen (5 bulls were higher with fresh semen and 4 were higher with frozen-thawed). In contrast, Vishwanath and Shannon (2000) reported that bulls generally followed the same fertility trend with fresh and frozen-thawed semen when optimum sperm numbers were used (2.5 million/dose for fresh and 20 million/dose for frozen-thawed) but when suboptimum sperm numbers were used (0.5 million/dose for fresh and 5 million/dose for frozen-thawed) fertility declined by 7 and 7.9% for fresh and frozen-thawed, respectively. More importantly, there was a significant bull by sperm number interaction, whereby some bulls dropped by over 20% when lower sperm numbers of frozen-thawed semen was inseminated and some bulls performed the same as with 20 million sperm. This trend of a greater variation among bulls at lower sperm concentrations was also observed by Den Daas *et al.* (1998) where maximum fertility for individual bulls was achieved at differing sperm concentrations and the sperm numbers needed to obtain 95% of the maximal conception rate ranged from 1 to 11 million sperm per dose. This highlights the variability in the susceptibility of an individual bull's semen to the freeze-thaw process and how freezing protocols should

be customised to individual bulls, an area of research that has received little attention in recent years. It also illustrates that individual bulls have different maximum fertility and the number of sperm required to achieve this varies among bulls.

In order to minimise differences in pregnancy rates among individual sires, over-compensation of sperm numbers typically occurs in the preparation of frozen-thawed semen, resulting in a sperm concentration that considerably exceeds the number of sperm necessary for maximum fertility. Thus, the 'true fertility' potential of a bull in the field is masked by the greater sperm number per insemination dose and this needs to be considered when attempting to understand the variation in SCR using *in vitro* assays. For example, consider an AI centre which processes semen at 15 million sperm per inseminate (as is typical) with an overall mean calving rate across all its bulls of 53%. A comparison of two of their bulls with a calving rate of 60% would lead to the conclusion that both bulls were of 'high fertility' and in any retrospective 'prediction type analysis', they would be treated as such. However, now consider that if assessed at a lower sperm number, one bull would have had the same fertility at a dose of 5 million sperm per straw while the second bull would have required 12 million sperm for this level of fertility. As the only fertility data available to the AI centre was at a concentration of 15 million sperm, both bulls would be considered to have the same fertility phenotype yet there are distinctive differences in the ability of their sperm to establish a pregnancy after the freeze-thaw process. Therefore, fertility can only be actually determined under conditions where sperm numbers are limiting (Hammerstedt, 1996) and it is not surprising that there is difficulty in identifying the causes of bull subfertility when a dubious fertility phenotype is used at the start. Ideally, the number of sperm at which a bull's fertility reaches a plateau should be determined,

but tracking semen straws with varying sperm concentrations in the field poses major logistical issues for most AI companies.

Where does reproductive wastage occur in bulls that vary in their sire conception rate following artificial insemination?

There have been a plethora of publications on the prediction of sire fertility using sperm functional (Sellem *et al.*, 2015), molecular (Rahman *et al.*, 2017) and genomic (Puglisi *et al.*, 2016) models as well as combinations of these. Despite this, there is still no single test, or combination of tests, which can reliably predict bull fertility. Very few studies have focused on attempting to understand why bulls whose semen has normal post-thaw motility and morphology, as viewed under a microscope, can still vary in fertility by up to 20% points. For most commercial situations, fertility is defined as cows either failing to return to oestrus (non-return rate) or confirmed pregnant by means of ultrasound scan, rectal palpation, blood progesterone or a calving event. These estimates of pregnancy status following insemination are incapable of differentiating the reasons for pregnancy failure. What is clear is that semen is deposited into the uterine body and the chance of a pregnancy varies among bulls. The possible reasons for this are presented in Figure 2 and the associated published studies are then discussed.

Sperm proteome and its relationship to the establishment of pregnancy

During ejaculation sperm become coated in proteins immediately during ejaculation that are secreted from the epididymides as well as the accessary glands and even though bull semen is typically diluted 15-25 fold during semen processing, the effects

of seminal plasma proteins are likely to be maintained as they adhere to sperm rapidly upon ejaculation. Numerous studies have focused on characterising the proteomic composition of seminal fluid (which also contains epididymal fluid) across a range of species (Druart *et al.*, 2013) and related these to fertility. Some of the seminal plasma proteins that have been positively related to bull fertility include osteopontin (Ca2+-binding protein) and lipocalin-type prostaglandin D synthase (Cancel *et al.*, 1997), telomeres-1 protein (POT1) (Aslam *et al.*, 2014) while other seminal plasma proteins have been negatively correlated to fertility including prostaglandin E2 receptor EP3 (PTGER3) (Aslam *et al.*, 2014). More functional studies are required to validate these and to characterise how exactly they influence the establishment of pregnancy.

Other studies have mapped the proteome of bull sperm and have reported correlations between specific proteins and sperm motility, morphology as well as fertility (D'Amours *et al.*, 2010). Sperm proteins can be broadly categorised into energy-related, structural and other functional proteins and sperm-bound proteins from bulls of varying fertility have been related to spermiation and energy homeostasis, membrane function, sperm-egg interactions and cell cycle regulation as well as glycolysis, post-translational changes during sperm maturation, capacitation and protection against oxidative stress, to name but a few (Gaviraghi *et al.*, 2010, Park *et al.*, 2012). Molecular defects in some of these proteins have been reported to be associated with low fertility or in certain cases, infertility. Somashekar *et al.* (2017) investigating sperm proteomic signatures regulating sperm function and fertility reported calmodulin (CALM1), spermadhesinZ13 (SPADH2), and phosphatidylethanolamine-binding protein 4 (PEBP4) to be present in higher amounts on sperm of high fertility bulls with PEBP4 being absent in infertile bulls. An earlier study by the same group reported that the seminal plasma protein PDC-109 was more abundant on sperm from low fertility bulls

(Somashekar *et al.*, 2015). The exact role of many of these proteins in bull fertility is still unclear and thus the current challenge for reproductive biologists is to move from lists of identified proteins to informed understanding of biological function given that they control key physiological events in the female tract.

Sperm communication and interaction with the female tract

The immunological responses to sperm and seminal plasma in the female tract are of considerable interest as these processes influence sperm capacitation, transport, selection, fertilisation as well as early embryo development (Schuberth *et al.*, 2008). The local immune responses of the epithelial lining, regulated by its secretions, constitute the main part of the mucosal innate immunity inside the uterus and oviduct which is largely mediated by cytokines, chemokines and prostaglandins (Bulek *et al.*, 2010). Much of the focus in humans and rodents has been on the bioactive signalling agents in seminal plasma and how they evoke gene expression and cellular changes in the innate immune system (see review by Schjenken and Robertson, 2014). The presence of sperm, seminal plasma and semen diluent causes a triggering of the first line of defense against foreign cells through increased production of pro-inflammatory cytokines, leading to an influx of polymorphonuclear neutrophils (PMNs) into the lumen of the female tract (Marey *et al.*, 2014). Polymorphonuclear neutrophils have been reported to clear dead and immotile sperm but also motile sperm (Li and Funahashi, 2010) and the presence of activated phagocytes can lead to decreases in sperm motility due to increased production of reactive oxygen species (ROS). Oxidative stress is known to be a major factor regulating the vitality and functionality of sperm; however, the precise implications of increased ROS within the female tract are not well understood . Sperm from bulls with below average fertility had significantly greater

ROS production compared to above average fertility bulls (Kumaresan *et al.*, 2017) which has in turn been related to increased deleterious effects of lipid peroxidation on the membrane and DNA integrity (Koppers *et al.*, 2011).

Using an *in vitro* model, Marey *et al.* (2016) demonstrated that endothelin-1 may be involved in supporting bull sperm survival until fertilisation through the protection of sperm from phagocytosis by PMNs in the bovine oviduct. In the macaque, beta-defensin 126 (BD126) has been reported to protect sperm from immune-recognition and binding of anti-sperm antibodies (ASA; Yudin *et al.*, 2005). Anti-sperm antibodies are produced in response to antigens present on sperm and can account for reduced sperm viability but also higher sperm mortality in the female reproductive tract (Rossato *et al.*, 2004). It is estimated that ASA are responsible for as much as 40% of unexplained fertility cases in humans and recent studies have also revealed a high level of ASA in both serum and seminal plasma from bulls that have a negative effect on their fertility through the prevention of capacitation (Zodinsanga *et al.*, 2015).

β-defensins glycoproteins coat sperm and have been identified as having a role in modulating the inflammatory response to enhance sperm survival (Yudin *et al.*, 2005). While the function of β-defensins in reproduction have not, until recently, been explored in farm animal species, knockout of a β-defensin gene cluster in male mice resulted in complete sterility (Zhou *et al.*, 2013). In men, variation in the *BD126* sequence contributes to subfertility (Tollner *et al.*, 2011) while the BD126 peptide has been reported to mediate sperm binding to the oviductal epithelium (Tollner *et al.*, 2008). Our group has reported bovine beta-defensin 126 (*BBD126*) to be extensively expressed in the reproductive tract of the bull with preferential protein expression in the cauda epididymis (Narciandi *et al.*, 2011) and on sperm (Narciandi *et al.*, 2016) with similar binding patterns on the sperm surface to macaque. Beta-defensin 126

increases the net negative charge on sperm (Tollner *et al.*, 2012), increases sperm motility, mucus penetration *in vitro* (Fernandez-Fuertes *et al.*, 2016) as well as sperm binding to oviductal epithelium *in vitro* (Lyons, 2016). We have also recently characterised the genetic variation in bovine β-defensin genes as well as completing the first whole-exome sequencing of AI bulls of divergent fertility (Whiston *et al.*, 2017). This dual approach successfully identified novel variants in both *beta-defensin* and *FOXJ3* genes as potentially regulating SCR through differential oviductal binding ability, as assessed *in vitro* (Whiston *et al.*, 2017). Using a microarray-based approach, Legare *et al.* (2017) characterised the expression of genes along the caput, corpus and cauda epididymis in bulls which differed in SCR. The transcriptional profiles between sub-fertile and fertile bulls clustered most closely in the cauda and corpus segments, whereas the profiles in the caput segment were distinct between sub-fertile and fertile bulls. Of the differently expressed genes, 10 were related to reproductive function and 5 were associated with the defense response (of which 2 belonged to the defensin family, namely *DEFB119, DEFB124*). Bulls carrying mutations in genes which encode these immunoregulatory peptides could produce sperm of higher immunogenicity which could well contribute to reduced sperm survival in the female reproductive tract and subfertility.

During ejaculation, binder of sperm proteins (BSPs) are secreted by bovine seminal vesicles into seminal plasma and immediately absorbed onto sperm (Leahy and de Graaf, 2012). Of these, BSP1, BSP3, BSP5 have been reported to facilitate uncapactitated bull sperm in binding to the epithelial lining of the uterotubular junction and isthmus, forming a sperm storage reservoir (Hung and Suarez, 2012). However, during the completion of capacitation, changes in their composition on sperm play a role in releasing sperm from these storage reservoirs. The ability of sperm to bind to

296 the oviductal epithelium appears critical to establishing a viable sperm population in
297 the oviducts and may aid in overcoming any asynchrony between the timing of AI and
298 ovulation. A number of studies have investigated the interaction between sperm and
299 the oviductal epithelium *in vitro*, and demonstrated that it is mediated by fucose
300 (Lefebvre *et al.*, 1997). Previous *in vivo* work has reported that the timing of
301 insemination is more important for low and average fertility bulls compared to high
302 fertility bulls (Macmillan and Watson, 1975) suggesting that there is a reduced ability
303 of sperm from low fertility bulls to develop a reservoir of functional sperm at the utero-
304 tubular junction and in the oviducts. Interestingly, Yousef *et al.* (2016) reported that
305 bovine oviductal epithelial cells provide an anti-inflammatory environment and the
306 sperm-epithelial binding further strengthens this, leading to the suppression of PMNs
307 in the bovine oviduct. In addition to facilitating sperm binding, Lessard *et al.* (2011)
308 investigating the etiology of idiopathic infertility in a beef bull established that his sperm
309 were unable to undergo the acrosome reaction, when induced using calcium
310 ionophore, and related this to the level of BSP1 that was much greater on sperm from
311 the infertile bull compared to that of his sire.

312 It is clear that there is cross-talk between semen and the female tract, starting in the
313 uterus with the induction of an inflammatory response and continuing in the oviduct
314 through sperm binding and subsequent release. There is evidence that bulls vary in
315 their capacity to complete these physiological processes and indications are that this
316 is related to the surface proteome of sperm that is influenced by both the epididymal
317 secretome and the composition of seminal plasma. The focus therefore should be on
318 characterising these parameters from bulls of divergent fertility with a view to
319 identifying key biomarkers (not just proteins), which can then be used in functional

studies to better understand how these regulate the dialogue between sperm and female reproductive tract.

Sperm transport in the female tract

In vivo assessments, either by flushing sperm from the segments of the reproductive tract following AI or using confocal imaging of sperm in the female tract as has been performed in sheep (Druart *et al.*, 2009), are unlikely to be sensitive enough to detect differences among bulls with varying SCR. Other approaches such as mucus penetration tests, assessment of accessory sperm number following AI and heterospermic insemination have been used to understand why some males sperm may be better able to navigate the female reproductive tract and its secretions than others.

The use of mucus penetration assays *in vitro*, which assess the ability of the sperm to travel through a capillary filled with artificial mucus or cervical mucus from oestrus cows has been used as a proxy for assessment of sperm transport and has been correlated to SCR (Al Naib *et al.*, 2011). While these studies were conducted in a static mucus environment, the development of microfluidic systems enables the characterisation of sperm rheotaxis, a phenomenon whereby sperm swim against a flow (Miki and Clapham, 2013) and offers a way of assessing sperm migration ability. While chemotaxis may guide sperm towards the ovulated oocyte once it is in its vicinity in the ampulla, rheotaxis has been proposed as a long-range guidance cue for sperm navigation along the female tract. Rheotaxis requires rotation of the sperm, which requires CatSper calcium-selective ion channels**.** CatSper glycoproteins form the sperm-specific voltage-gated Ca^{2+} channels localised along the membrane of the

sperm flagellum. CatSper channels contain glycoproteins that are involved in positioning regulation and recent work by our group has demonstrated that hyperactive bull sperm exhibit an increased rheotaxis response (Johnson *et al.*, 2017). Targeted disruption of CATSPER 1, CATSPER2, CATSPER3 or CATSPER4 inhibits hyper-activated motility and thus rheotactic reponse (Johnson *et al.*, 2017). There are no published studies on the rheotactic response of bulls differing in SCR. The advent of 3-D printing will no doubt facilitate the development of more physiological and sensitive models for studying this as well as sperm interaction with the female tract and its secretions.

Heterospermic insemination involves the insemination of the semen mixture at the one point in time into the same female and thus levels the playing field. Each sperm should have an equal chance to reach and fertilise the oocyte without influence by technician, cow age/parity/genetic merit/days in milk, timing of insemination relative to oestrus, season and management (Beatty *et al.*, 1969). For this reason, it has been reported that heterospermic insemination is up to 170 times more sensitive in ranking reproductive outcome than homospermic measures (Flint *et al.*, 2003). To put this into context, homospermic insemination requires thousands of inseminations to compare fertility of two males accurately while heterospermic insemination has been reported to be able to test the fertility of a bull accurately and rapidly using fewer than 100 females. Overstreet and Adams (1971) inseminated a mixture of equal numbers of labelled and unlabelled rabbit sperm from two bucks and flushed the reproductive tract of does 6 or 13 h later for evidence of selective transport and sperm viability. The numbers of sperm from each male in each of the segments of the reproductive tract were equal at 6 h, but by 13 h sperm from the superior buck predominated in the uterus and oviducts. More sperm from the superior buck were attached to the zona pellucida

and fertilised more oocytes. When semen was place in the oviducts the sperm were present in equal numbers in the vicinity of the oocyte but the skewed proportion of offspring and labelled sperm penetrating the oocyte still favoured the superior buck. Using heterospermic insemination of fluorescently-labelled sperm, Ferreira (1972) reported that sperm number recovered from the vagina, uterus and oviduct was similar among males, as was the number of sperm bound to the zona pellucida of recovered oocytes. These observations lead the authors of these aforementioned studies to conclude that sperm are present in equal numbers in the immediate vicinity of the oocyte and perhaps rate of oocyte penetration or subsequent activation of the oocyte differed among males. This is also in agreement with Macmillan and Watson (1975) who reported that all bulls have a similar opportunity to fertilise when AI occurred close to ovulation, but when AI occurred at longer intervals prior to ovulation, the sperm of some bulls, which were obviously present at longer intervals to AI, were no longer alive or capable of fertilising an oocyte. These studies emphasise the importance of having a population of functional sperm in the oviducts at the time of ovulation.

Fertilisation and early embryo development

Fertilisation success following AI in cattle with semen from high fertility sires is in the order of 90-95% in heifers and moderate yielding cows (Diskin and Sreenan, 1980). A meta-analyis by Sartori *et al.* (2010) estimated that fertilisation rates in North American high-producing Holstein cows to be 83% while pregnancy rates of similar genetic merit lactating cows to be 33%. Several studies have been performed over the last 30 years in which cows have been slaughtered at various time-points post insemination in order to assess embryo viability. The majority of this reproductive wastage in single-

393 ovulating cows had been attributed to early embryo loss with <50% of recovered
394 embryos from high yielding lactating cows viable 7 days after AI followed by additional
395 losses through Day ~34 (Sreenan and Diskin, 1986). Sartori *et al.* (2002)
396 demonstrated that lactating cows had poorer quality Day 5 embryos than both heifers
397 and dry cows but surprisingly more accessory sperm indicating that delayed sperm
398 transport was not a causative effect. In single ovulating cows, most embryos and
399 approximately 80% of unfertilised oocytes had at least one accessory spermatozoon
400 (Cerri *et al.*, 2009) while Sartori *et al.* (2002) reported mean values of 18–42 sperm in
401 embryos and 18 in unfertilised ova. All of these aforementioned studies were focused
402 on cow factors and there is a complete dearth of published studies focusing on the
403 relationship between SCR and the contribution of the sperm to failure of sperm
404 transport, fertilisation or embryo development. Using a small number of bulls with
405 below and above average fertility, Ortega *et al.* (2017) recently assessed the
406 contribution SCR to pregnancy establishment and reported that bulls with a higher
407 SCR had an advantage in terms *of in vivo* and *in vitro* production of embryos. In the
408 same study there was no effect of SCR on pre-implantation conceptus elongation and
409 development. Kumaresan *et al.* (2017) reported that bulls with below average fertility
410 had a significantly lower sperm population with intact acrosomes post thawing
411 compared to bulls with above average fertility, similar to earlier reports (Singh *et al.*,
412 2016).
413 *In vitro* fertilisation (IVF) is a powerful tool to assess the fertilising ability of sperm. The
414 kinetics of sperm penetration (Ward *et al.*, 2002) as well as the first cell cycle
415 (Comizzoli *et al.*, 2000) and of the first mitotic cleavage after fertilisation (Lonergan *et*
416 *al.,* 1999) are highly correlated with the likelihood of an embryo developing to the
417 blastocyst stage and to the quality of those embryos (Dinnyes *et al.*, 1999). Ward *et*

418 *al.* (2001) was able to discriminate between bulls of high and low field fertility based
419 on the timing of the first cleavage division post insemination *in vitro*, whereby embryos
420 fertilised from high fertility bulls cleaved first and significantly more of these early
421 cleaving zygotes were more competent in terms of development to the blastocyst
422 stage than those that cleaved later. The same study reported a significant correlation
423 between Day 7 blastocyst yield and field fertility while a separate study reported an
424 effect of SCR and cleavage rate (Al Naib *et al.*, 2011). In contrast, Kropp *et al.* (2017)
425 reported no differences in the morphology and development to the blastocyst stage
426 but preimplantation embryos derived from high and low fertility bulls displayed
427 significant transcriptomic differences, which they postulated could influence the
428 reprogramming of the early embryo. Therefore, the evidence suggests that a portion
429 (contribution will vary among sires) of the embryo death before approximately Day 8
430 is caused by the fertilising sperm but the specific aspect of the sperm causing this
431 effect is unclear.

432

433 *Role* of sperm deoxyribonucleic acid integrity *and methylation signature*

434 Individual bulls vary in the levels of sperm DNA fragmentation that they exhibit (Takeda
435 *et al.*, 2015) and there appears to be a growing link between this parameter and early
436 embryonic loss and even foetal development and health of the offspring (Evenson and
437 Jost, 2000). During spermiogenesis, sperm chromatin is remodelled whereby core
438 histones are replaced by transition proteins which are subsequently replaced by
439 protamines resulting in chromatin that is tightly compacted and resistant to
440 denaturation (Filho *et al.*, 2015). This compaction is necessary to protect sperm
441 chromatin during transit through the epididymis and female reproductive tract. Shortly

after fertilisation, sperm protamines are replaced by maternal histone variants. Thus, defects of sperm chromatin structure affect sperm function during fertilisation, first cleavage and early embryonic development. Inadequate sperm chromatin protamination and DNA integrity were associated with defects in bull sperm chromatin condensation, coinciding with reduced *in vivo* fertility (Dogan *et al.*, 2015). Disruption to defective chromatin packaging during spermiogenesis results in sperm that are susceptible to denaturation and there is growing evidence, that the status of sperm chromatin at the time of fertilisation can influence embryonic survival (Sakkas *et al.*, 2002).

A number of studies have demonstrated a relationship between the levels of DNA fragmentation in bull sperm and SCR (Kumaresan *et al.*, 2017) with a high level of DNA fragmentation correlated to sperm morphology (Nagy *et al.*, 2013) as well as to a reduced sperm fertilisation potential. DNA fragmentation values of between 7 – 10% have been reported to be indicative of low AI success in bulls as DNA damage can jeopardise embryonic development (Karoui *et al.*, 2012). However, it must also be noted that many bulls that have lower fertility do not always exhibit increased levels of DNA fragmentation as Rodriguez-Martinez and Barth (2007) reported no direct correlation of DNA fragmentation with fertility. Therefore, like many other *in vitro* parameters, DNA fragmentation seems more useful when using the negative biomarker approach whereby high levels indicate sperm defects but low levels do not guarantee fertility.

It is has been known for some time that, at the time of fertilisation, sperm deliver much more than just DNA, but rather an entire package including RNAs, transcription factors, and cell signalling molecules (Krawetz, 2005). While a number of studies have demonstrated that the transcriptome is significantly different among sires of varying

fertility (Feugang *et al.*, 2010) it has only recently been reported that the embryonic transcriptome is influenced by the "RNA package" delivered by sires of varying fertility status at the time of fertilisation (Kropp *et al.*, 2017). The same study characterised the epigenetic signature of the sperm between bulls of high and low fertility and revealed 76 regions to be differentially methylated between sires of divergent fertility. While cleavage and blastocyst rate was not affected, the resultant IVF-derived embryos had significantly different transcriptomic profiles with genes relating to metabolic processes and catalytic activities more highly expressed in sperm from high fertility bulls. Errors relating to the condensation of the DNA during spermatogenesis as well as maintenance of epigenetic marks could possibly explain the differences in embryonic gene expression. Indeed, lower levels of DNA condensation, protamine exchange, and higher DNA damage have been observed in sperm from lower fertility bulls in comparison to higher fertility bulls (Dogan *et al.*, 2015). In addition, a recent study focusing on the epigenetic profiles of young bulls highlighted that 10 month-old bulls have a different sperm DNA methylation pattern compared to both 12 and 16 month old bulls (Lambert *et al.*, 2018). Given the current trend of using semen from elite genomically-selected bulls, this study demonstrates that such bulls not only have poorer sperm motility and morphology but also an altered epigenetic profile that has the potential to influence embryonic development as well as the genotype and the phenotype of the subsequent offspring.

Sperm ribonucleic acid and its relationship to sire conception rate

Ejaculated sperm are "stripped-down" cells, equipped with a strong flagellum to drive them through an aqueous mucus environment but unencumbered by cytoplasmic organelles. As a result they are transcriptionally inactive but do retain remnant

messenger RNA (mRNA) that are left over from spermatogenesis that can be used for diagnostic purposes. Thus, transcripts (and translation) products of genes are present even in functionally mature ejaculated sperm but are products of later spermatids (or earlier) active gene expression, processes that cease before spermiation. Recent analyses are challenging this belief suggesting that the rich repertoire of coding and non-coding RNAs in sperm is not a haphazard remnant from spermatogenesis in the testes but a carefully selectively retained and functionally coherent collection of RNAs (Das *et al.*, 2013). More recent interpretations suggest human sperm retain mRNA that can be translated into protein in the oocyte after fertilisation (Jodar *et al.*, 2013). However, their precise role in the regulation of fertilisation and early embryonic development in the bovine remains to be determined. The mRNA expression of proteins associated with sperm function in bulls of high and low SCR reported a number of genes correlated with fertility status (Kasimanickam *et al.*, 2012). Feugang *et al.* (2010) analysed the RNA profiles of sperm from high and low fertility Holstein bulls using Affymetrix bovine genechips and reported differential expression in the abundance of mRNAs. A total of 415 transcripts out of approximately 24,000 were differentially detected in sperm collected from both fertility groups. Sperm from the low-fertility bulls were deficient of transcripts for transcriptional and translational factors while sperm from high fertility bulls contained higher concentrations of transcripts for extracellular space and membrane protein locations.

Short non-coding microRNAs (miRNAs) do not code for proteins but various studies have reported that miRNAs regulate gene expression and also play a major role in embryo development (Boerke *et al.*, 2007). However, their precise role in the regulation of fertilisation and early embryonic development in the bovine remains to be determined. miRNA profiling from high and low fertility bulls has also been

517 previously performed (Govindaraju *et al.*, 2012) with seven miRNAs (aga-3155, -8197,
518 -6727, -11796, -14189, -6125, -13659) being differentially expressed. Tscherner *et al.*
519 (2014) reported that the miR-34 miRNAs play a role in developing bovine gametes
520 and suggested that individual variation in sperm miR-34 family abundance may be a
521 biomarker of male bovine fertility. In addition, single nucleotide polymorphisms in
522 target mRNA or miRNA have revealed associations with traits of economic interest
523 and highlight the potential use of miRNAs in future genomic selection programs
524 (Fatima and Morris, 2013). Sperm miRNA may be useful in understanding the
525 transmission of epigenetic characteristics to male calves and its connection with
526 transgenerational inheritance of fertility/subfertility related traits. High-throughput RNA
527 sequencing approaches will aid in the determination of the key coding and non-coding
528 transcripts controlling sperm function and thus SCR.

529

530 **Future directions of research directed at understanding the etiology of**
531 **idiopathic bull fertility**

532

533 Male fertility has received far less attention in comparison to female fertility yet it is
534 undoubtedly complex and definitely multifactorial. Despite many positive findings, the
535 small numbers of bulls and, in some cases, an unreliable fertility phenotype due to
536 insufficient insemination records for individual bulls as well as issues around sperm
537 number used make interpretation of the findings of many studies challenging and
538 sometimes unrepeatable when applied to different datasets. Despite this, it is now
539 clear that that the sperm deliver not only DNA but also RNA and signaling factors to
540 the oocyte at fertilisation. The most fruitful avenues of further investigation would
541 appear to be around the differences among bulls in the kinetics of sperm penetration

as well as completion of the first cell cycle and of the first mitotic cleavage after fertilisation. Embryos that cleave first are most likely to successfully reach the blastocyst stage and the quality of these embryos is superior at the preimplantation stage than later developing embryos. The pathophysiology of delayed cleavage may reside with the non-coding RNAs and or alterations in epigenetic signatures within the sperm which are most likely to be altered during testicular development or by epididymal modifications. An in-depth examination of these factors may shed new light on the cross-talk between bovine sperm and the early stages of embryo development; and importantly how this may be perturbed in bulls of low fertility. Future studies will no doubt take advantage of recent advances in high-throughput techniques to study DNA, RNAs, proteins, lipids, glycans and metabolites in combination. These 'OMICS'-based technologies have increased our capacity to study new and novel aspects of sperm function and to get a broader view of these complex biological systems. They hold the main advantage of providing large volumes of information at relatively low cost and recent advances in bioinformatics enable the analysis and interpretation of large datasets in a more integrated systems biology approach.

Like so many studies thus far, these technologies will undoubtedly produce lists of biomarkers that are different between bulls of varying fertility. The major challenge then is to define which ones are physiologically important. For this, we need novel functional approaches comprising of both *in vitro* and *in vivo* methods. However, as outlined earlier in this review, before we go down this path we must be cognisant of the limitations of sire fertility estimates especially when inseminations are performed with high numbers of sperm. Then, we should ensure experiments are sufficiently powered with bulls across a wide range of the fertility spectrum in the quest to identify the reasons for the variation in SCR.

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Declaration of interest

The authors have no vested interests to declare.

Ethics statement

This manuscript is a review of existing published work and as such did not use animals for its' preparation.

Software and data repository resources

None.

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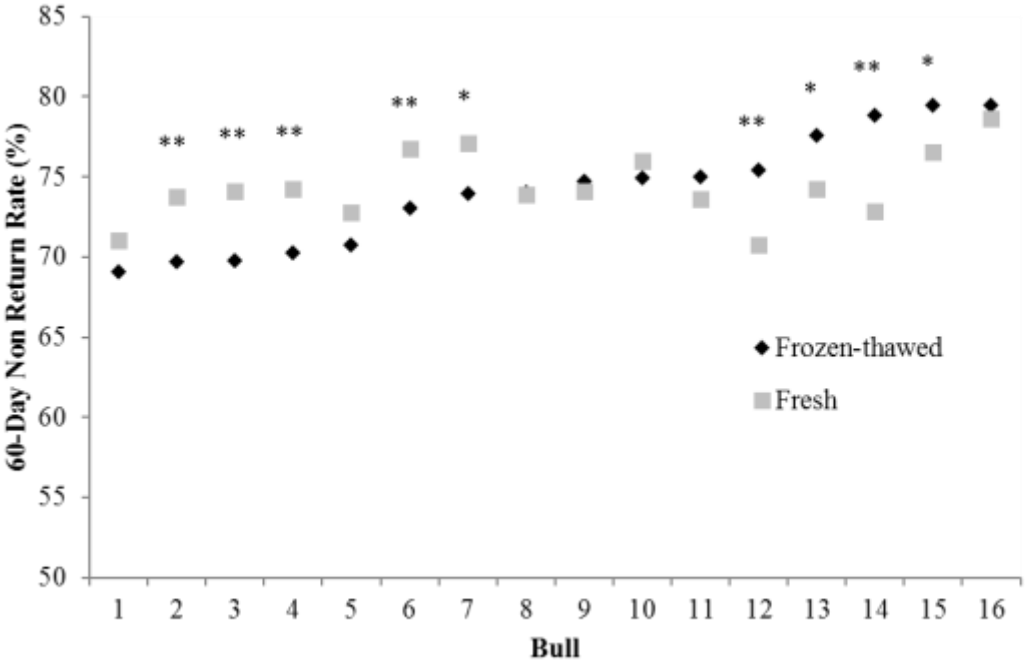

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851 **Figure 1**: Characterisation of the variation between 60 day non return rate in sixteen
852 bulls with split ejaculates used as fresh or frozen-thawed semen (n=66,252
853 inseminations). *P<0.05, **P<0.01 between semen type within bull.

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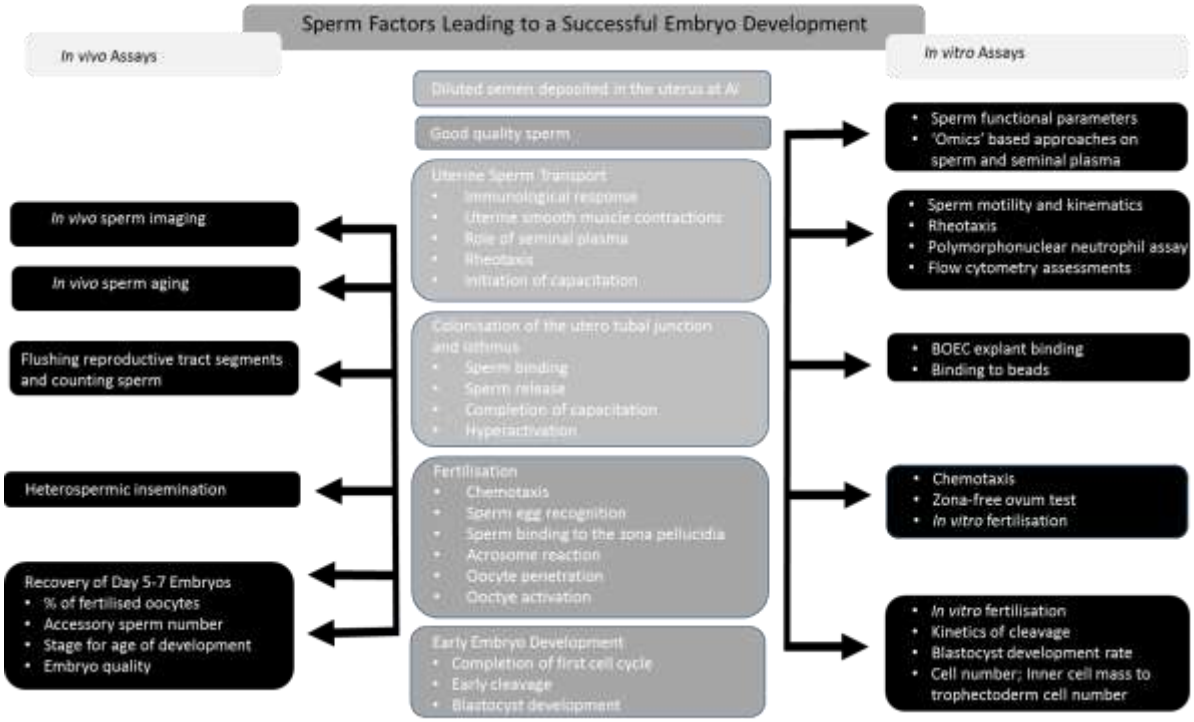

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864 **Figure 2.** Events leading to the establishment of a viable embryo following artificial
865 insemination (AI; Centre Column), with the *in vivo* (Left Column) as well as the *in vitro*
866 (Right Column) assessments that have been used in published studies to characterise
867 the differences in these events between bulls of varying fertility.

868