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The future of assessing bull fertility: Can the 'omics fields identify usable biomarkers?[†]

Erin K. Klein[1],*, Aleona Swegen[1,2], Allan J. Gunn[3,4], Cyril P. Stephen[3,4], Robert John Aitken[1] and Zamira Gibb[1]

[1]Priority Research Centre for Reproductive Science, University of Newcastle, Callaghan, New South Wales, Australia
[2]Nuffield Department of Women's and Reproductive Health, University of Oxford, Oxford, UK
[3]School of Animal and Veterinary Sciences, Charles Sturt University, Wagga Wagga, New South Wales, Australia
[4]Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, New South Wales, Australia
***Correspondence:** Priority Research Centre for Reproductive Science, University of Newcastle, University Drive, Callaghan, NSW 2308, Australia.
E-mail: erin.klein@uon.edu.au
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Abstract

Breeding soundness examinations for bulls rely heavily on the subjective, visual assessment of sperm motility and morphology. Although these criteria have the potential to identify infertile males, they cannot be used to guarantee fertility or provide information about varying degrees of bull fertility. Male factor fertility is complex, and the success of the male gamete is not necessarily realized until well after the spermatozoon enters the oocyte. This paper reviews our existing knowledge of the bull's contribution from a standpoint of the sperm's cargo and the impact that this can have on fertilization and the development of the embryo. There has been a plethora of recent research characterizing the many molecular attributes that can affect the functional competence of a spermatozoon. A better understanding of the molecular factors influencing fertilization and embryo development in cattle will lead to the identification of biomarkers for the selection of bulls of superior fertility, which will have major implications for livestock production. To see this improvement in reproductive performance, we believe incorporation of modern technology into breeding soundness examinations will be necessary—although many of the discussed technologies are not ready for large-scale field application. Each of the 'omics fields discussed in this review have shown promise for the identification of biomarkers of fertility, with certain families of biomarkers appearing to be better suited to different evaluations throughout a bull's lifetime. Further research is needed for the proposed biomarkers to be of diagnostic or predictive value.

Summary Sentence
A review of proteins, transcripts, and metabolites identified in spermatozoa and seminal plasma, with a focus on molecular factors correlated with high or low fertility bulls to evaluate if they could be used as predictive biomarkers in the future.

Keywords: bull fertility, sperm, seminal plasma, fertility marker, biomarker

Introduction

When the spermatozoon penetrates the oocyte, whether it arrived there naturally or with the aid of assisted reproductive technology, it brings with it all of the baggage of its life up until that point. This baggage includes molecular factors, such as proteins [1], RNA species [2, 3], and metabolites [4], and the intrinsic damage to these factors caused by environmental stressors during cell development [5] and/or storage [6].

The mammalian spermatozoon is a highly specialized cell with the sole purpose of fertilizing an oocyte. At its most basic, it consists of a head, where the cell's haploid genetic cargo is tightly compacted in the nucleus, a midpiece harboring mitochondria, and a tail that facilitates propulsion [7]. Also located in the midpiece is the centriole, which is essential for normal embryonic development as the sperm-contributed centriole orchestrates all cell division in the progeny [8]. Along with DNA, the spermatozoon contains coding and non-coding RNA (ncRNA), as well as an array of proteins, lipids, carbohydrates, and small-molecular-mass metabolites associated with cell function [9]. At the conclusion of

spermatogenesis, spermatozoa appear to be structurally complete but are not yet capable of natural fertilization, gaining this ability following epididymal transit [10]. Upon ejaculation, spermatozoa are transported in seminal plasma; a mixture of secretions from the testes, epididymis, and accessory sex glands.

During natural copulation, bull spermatozoa are deposited in the vagina of the cow. As this is a considerable distance from the site of fertilization, it is a requirement that spermatozoa be sufficiently motile to travel to the oviduct and locate the oocyte for fertilization to occur [2]. Because of this, low sperm motility is one of the major reasons for male subfertility or infertility [11]. Along with a physical examination and scrotal circumference measurement, assessments of sperm motility and morphology are key parts of the bull breeding soundness examination (BSE) [12].

The Society for Theriogenology in the USA has had protocols for conducting BSEs since at least 1956 when they were known as the Society for the Study of Breeding Soundness of Bulls. These procedures have been updated multiple

times in the more than half a century that has followed, but changes have largely been to the forms and terminology used or to requirements to pass with relatively minor changes to the examination process itself [12–14]. Similar pre-breeding examinations are recommended by the Australian Association of Cattle Veterinarians [15], the British Cattle Veterinary Association [16], the Western Canadian Association of Cattle Practitioners [17], and the South African Veterinary Association [18]. It has been estimated that one in five bulls has inadequate semen quality and/or physical soundness to pass these BSEs [19]. Certainly, BSEs are useful in identifying more obvious cases of infertility or subfertility; however, sperm fertility is a complex multifactorial faculty which cannot be accurately assessed solely by visual examination [20]. High motility and normal morphology scores do not guarantee fertilizing ability and bulls that pass these requirements can fail to produce offspring [21, 22]. Therefore, it is evident that other factors must also contribute to fertilization and normal embryo development. It is difficult to estimate the percentage of breeding bulls with subfertility, as many go unidentified, but unidentified subfertile bulls can cause substantial economic loss with poor conception rates resulting in late season pregnancies and/or producers wrongly culling cows over bull fertility issues. With a half a century's worth of new technology and advancements in knowledge available, BSEs will need to incorporate emerging molecular tools if we are to see further improvements in reproductive efficiency.

True reproductive efficiency is determined not only by fertilizing capabilities but also the ability to support embryo development. While considerable research has been done on assessing fertilization potential based on sperm characteristics, including motility [23], plasma membrane and acrosome integrity [24, 25], and DNA structural integrity [26], less is known about paternal influence on the development of a healthy, viable embryo. As early embryo mortality (prior to day 24) likely accounts for 75–80% of all embryo and fetal mortalities in cattle [27, 28], this is a key area for improving the number of healthy offspring. Much of embryo development is reliant on the competence of the oocyte [29, 30]; in the human, we know that the male gamete can also be responsible for numerous developmental abnormalities [31], and it is reasonable to assume that the same would be true for all mammalian species, including cattle.

In recent years, high-throughput technologies have emerged as a way to investigate molecular components, including proteins by proteomics [32], RNA by transcriptomics [33], and metabolites by metabolomics [34]. Through the analyses of these factors, in both spermatozoa and seminal plasma, correlations have been made between high and low fertility males and the presence, absence, over- or underexpression of certain components, which will be discussed within this review. With these discoveries comes the potential for determining a male's fertility status prior to breeding, whereas historically, fertility has had to be proven through the siring of offspring.

This paper reviews recent research characterizing the molecular attributes of bull semen, with particular attention to research correlating these molecular attributes to fertility status. The identification of biomarkers and their use to predict male fertility would have a powerful impact on production animal industries where higher conception rates and successful pregnancies translate directly into increased profits.

Maintenance costs would also be reduced as infertile or subfertile bulls could be removed from the herd much earlier, and genomic selection may be able to shorten generation intervals, as it has done in the dairy industry [35].

Proteins

Proteins are the workhorses of biology, being largely responsible for the phenotypes encoded by the genetic script, the structure and function of cellular machinery, and the interactions between receptors and other cellular components. The advent of proteomics—the study of large protein populations representative of an entire biological component, fluid or physiological state—has provided insight into male fertility across multiple species [36–38]. The seminal proteome comprises proteins from the sperm cells themselves and from accessory sex gland secretions. When spermatozoa leave the testes they are not yet fertile, acquiring fertilizing ability in part from proteins in the epididymal fluid [39].

In the mid-1990s, three studies by Bellin et al. found a positive correlation between the presence of heparin-binding proteins (HBP) and bull fertility potential. The first study identified the heparin-binding complex HBP-B5 in sperm membranes and seminal plasma [40]; the second examined the HBP-B5 proteins (HBP-30, HBP-24, and HBP-21.5) individually in sperm membranes and seminal plasma [41]; and the third focused specifically on HBP-30, also called fertility-associated antigen (FAA), in sperm membranes [42]. In the third study, selecting FAA-positive bulls for breeding resulted in an increased number of pregnancies, with pregnancies occurring earlier in the breeding season [42]. In characterizing these proteins, they found the HBPs to be similar to the DNase I-like protein family [43].

Early gel-based studies also linked lipocalin-type prostaglandin D synthase (PTGDS) [44], phospholipase A2 [45], osteopontin (SPP1) [45], and P25b [46] to higher fertility in the bull. To date, the majority of the proteomic research has been done using human spermatozoa, where thousands of proteins have been identified and characterized [47]. Often assumptions are made on the role of a protein in one species using analogous proteins from other species. The analogs of P25b in human and hamster, P34H and P26h, respectively, are involved in recognition of the zona pellucida [48, 49], suggesting that P25b may have the same role in bovine spermatozoa and that it would be logical for a lower abundance of this protein to have a negative effect on fertilization.

The binder of sperm proteins (BSPs), previously known as bovine seminal plasma proteins, are another protein family worth noting. BSPs account for nearly 70% of the protein content of bovine seminal plasma [50], although mixed reviews exist on their benefit or detriment to fertility. The BSP proteins, BSP3 [51] and BSP5 [45], were found in higher concentrations in subfertile bulls, which may be explained by increased concentrations of BSPs inducing cholesterol efflux, resulting in damage to sperm membrane and premature capacitation-like changes [52]. Proteins from the BSP family were found to be upregulated following scrotal insulation [53], suggesting that this is one of many ways in which bull fertility is affected by heat stress [5]. While an overabundance of BSPs can be detrimental, they are an important component of seminal plasma and aid in fertilization by facilitating

binding of spermatozoa to the oviduct epithelium and maintenance of motility during storage in the oviduct [54] and may aid in sperm membrane protection during cryopreservation [55].

In the first comprehensive proteomic analysis of bovine spermatozoa, 2051 proteins were reported to be unique to highly fertile bulls, 2281 proteins unique to low fertility bulls, and 125 differentially expressed between the two groups [1]. Of these, only about 15% had been previously described, and the identification of most of those were predicted based on similarities to proteins identified in other species. As the popularity of bovine sperm proteomics continues to grow, more studies using high-throughput methods have found correlations between protein abundance in spermatozoa [56–59] and seminal plasma [59–61] of high and low fertility groups; the more abundant proteins identified in spermatozoa, seminal plasma, or both are shown in Figure 1A. These proteins have known or assumed roles in many aspects of fertility, from spermatogenesis to embryo development, as depicted in Figure 1B. Supplemental Table S1 provides additional details on the identified proteins and their likely roles related to fertility as well as the method of identification. While the identified proteins correlated to fertility found in seminal plasma were split evenly between high and low fertility, virtually all of the upregulated proteins from spermatozoa alone were higher in high fertility bulls (Figure 1). The vast majority of proteins found in spermatozoa have roles in the earliest stages of the sperm's journey: spermatogenesis, maturation, and energy production (Figure 1). Of the previously mentioned proteins, all three BSPs, SPP1, and PTGDS were again identified in association with fertility status, though here PTGDS was significantly correlated to low fertility [61].

Earlier studies into the bull sperm proteome were performed using cryopreserved straws of semen [56, 57], while more recent studies tend to separate seminal plasma from spermatozoa prior to snap freezing [58, 59, 61] or lyophilization [60]. Significant changes in abundance of proteins in both spermatozoa and seminal plasma can occur during cryopreservation, interestingly increasing the abundance of some proteins [62]. More expectedly, cryopreservation has been shown to decrease the amount of sperm-bound proteins, specifically BSPs [50], which could be explained by BSP proteins binding to a lipoprotein component of egg-yolk extenders [52]. While this may be beneficial for protecting the sperm membrane during storage, the use of protein-containing extenders likely interferes with proteomic analysis. It will be necessary to confirm that these same markers can be identified as differentially expressed in fresh samples for them to be usable biomarkers.

Proteins in spermatozoa and seminal plasma show great potential as predictive biomarkers of fertility, but as with so many factors involved in fertility, these biomarkers may be better suited to identifying and removing infertile and subfertile bulls from the breeding herd, leaving bulls with a likelihood of higher fertility. Some proteins, such as PEBP4, have been found to be absent in infertile bulls and low or absent in low fertility bulls [58]. For proteins to be a reliable indicator of fertility, a combination of markers will need to be investigated. A combination of four proteomic markers previously identified [63] was used to predict bull fertility [64]. Although they found no meaningful change in the overall accuracy of the prediction between the combined assay and use of the single protein marker enolase 1, sensitivity and

negative predictive value reached 100% when all four markers were used.

RNA

In addition to proteins, spermatozoa also deliver both coding messenger RNAs (mRNAs) and ncRNAs to the oocyte [65]. As spermatozoa are transcriptionally and translationally silent, sperm RNAs were previously believed to be inert remnants of spermatogenesis but may be vital to the conveyance of the paternal genome [66]. The importance of sperm-derived mRNAs in the process of embryo development has been demonstrated using mouse models. Sperm-derived mRNAs have been found within embryos, remaining intact through the first cleavages [67], thereby suggesting a role in post-fertilization embryo development, and the removal of the sperm mRNAs leads to a significant reduction in blastocyst development and live birth rates [68].

In the bull sperm transcriptome, correlations have been found between certain mRNAs and motility [69] and have been shown to be distinctly different between bulls of high and low fertility [2, 3]. Some RNAs may indeed be remnants from spermatogenesis, but their presence or absence could still be an indication of the success or failure of necessary spermatogenic events [70].

Usually categorized as long non-coding RNAs and small RNAs (sRNAs), ncRNAs help regulate post-transcriptional gene expression. Studies using mouse models have discovered that the sperm sRNA profile is remodeled during epididymal transit and maturation, with a loss of piwi-interacting RNA and a gain of tRNA fragments; this remodeling and acquisition of sRNAs is essential for proper embryo development [71, 72].

Recent investigations into the differential expression of micro RNAs (miRNAs) in bulls have explored variations in fertility [73] and sperm motility [74]. In one study, seven miRNAs were found to be differentially expressed between bulls of moderate and high fertility, with a greater expression of these miRNAs in the moderate fertility bulls and two of the miRNAs undetectable in the high fertility group [73]. Another study identified differentially expressed miRNA of high and low motility sperm populations obtained from single ejaculates, separated post-thaw by density gradient centrifugation [74]. Although there was no significant difference between the expression of the five most highly abundant miRNAs of moderate and high fertility bulls [73], four of these miRNAs were significantly differentially expressed based on motility [74], with bta-miR-20a, bta-miR-15b, and bta-miR-93 higher in the high motility fractions and bta-miR-100 higher in the low motility fractions. Of the seven miRNAs that were significantly differentially expressed between moderate and high fertility groups [73], only two (bta-miR-34c and bta-miR-19b) were found to be more abundant in the high motility fractions [74].

Both of these studies used frozen–thawed semen which could confound the results, as cryopreservation has been shown to affect the transcriptomic profile of spermatozoa [75]. Shangguan et al. found that 55 miRNAs were differentially expressed between fresh and frozen spermatozoa, with 31 downregulated and 24 upregulated in fresh spermatozoa [75]. The GO and KEGG pathway analysis of the genes targeted by miRNAs and mRNAs differentially expressed
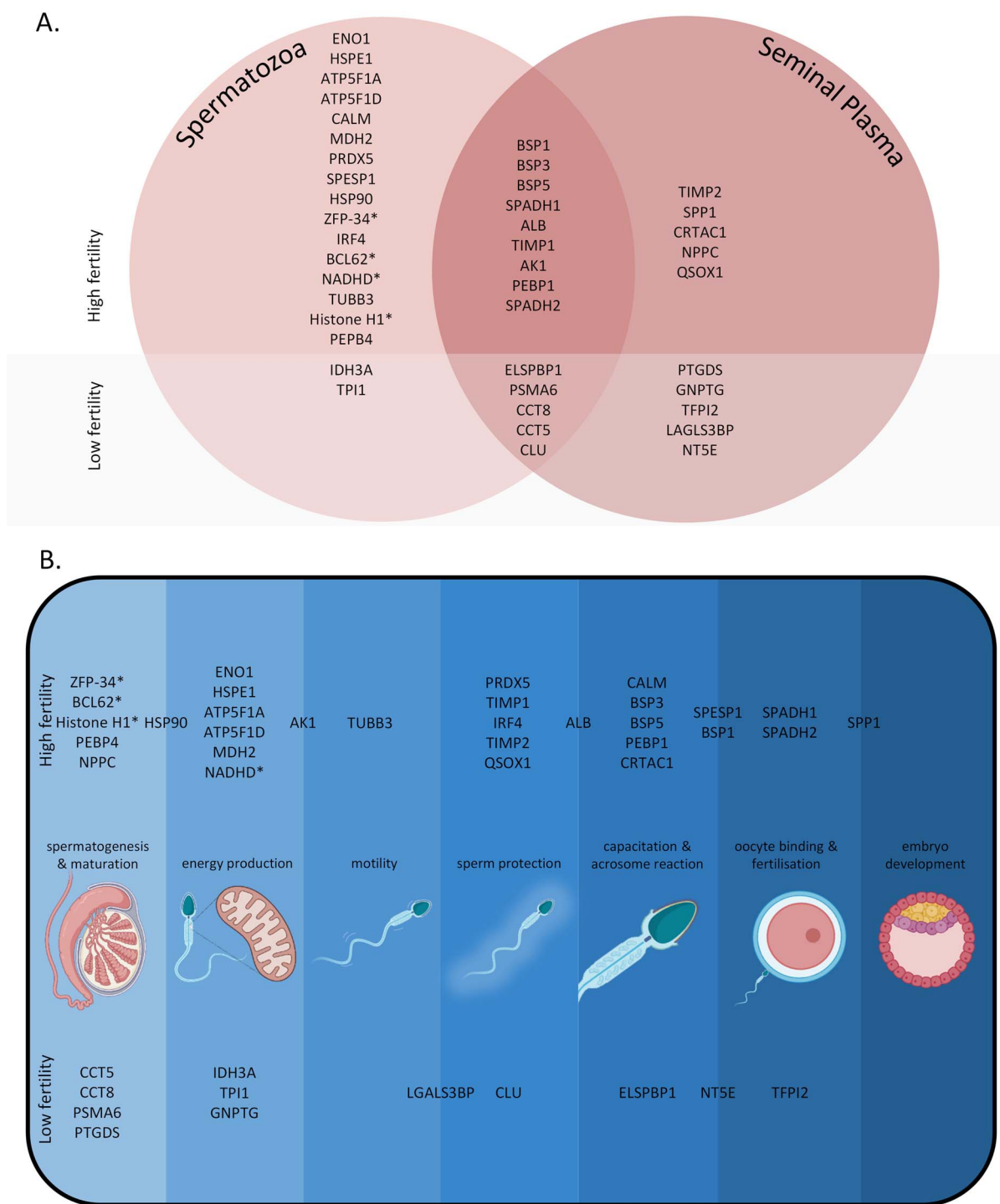
Figure 1. Proteins identified in spermatozoa and seminal plasma of bulls correlated with fertility status have many different roles in the series of events leading up to fertilization and embryo development. (A) Proteins found in spermatozoa [56–59] and seminal plasma [59–61] of bulls have been linked to high or low fertility status. (B) These identified proteins are shown where they are most likely to impact fertility. *Abbreviation used by reference is not a gene name (for more details, see Supplemental Table S1).

between fresh and frozen spermatozoa in the study revealed genes with functions related to apoptosis as well as fertilization processes and ATP generation [75]. The alteration of miRNA expression of sperm cells by cryopreservation, including the enrichment of functions related to apoptosis or cell death, could result in the low fertility of the spermatozoa or even influence epigenetic reprogramming or apoptosis in the embryo [76]. Sperm-borne miRNAs have also been found

to be differentially expressed between different breeds of bulls [77], though as semen quality parameters also vary across breeds [78], this should be expected.

Sperm RNAs are believed to serve as a mode of epigenetic inheritance, i.e., the inheritance of acquired traits. Research in mice has demonstrated that stress and diet can affect RNA expression transgenerationally. In recent studies, behavioral traits related to depression [79] and chronic stress [80] were

conveyed through the microinjection of sRNAs purified from spermatozoa of traumatized males into fertilized oocytes, providing evidence that sperm sRNAs are capable of passing on acquired traits to offspring. Paternal high-fat diets and paternal exercise have also been linked to changes in sperm RNA expression [81]. Many questions still exist as to how embryonic development is influenced by sperm sRNAs and how traits caused by sRNAs in spermatozoa are then transmitted to subsequent generations, but it has been suggested that spermatozoa may be receiving these sRNAs from somatic cells during development via epididymosomes—small extracellular vesicles in the epididymis [82–85].

As transcriptomics is a relatively new field of study, RNA biomarker tests still appear to be a long way from predictive application. One of the major obstacles to identifying RNA biomarkers is in the low quantity [86] and quality [69] of RNA extracted from spermatozoa. The RNAs are unstable and need specialized equipment for analysis and sequencing, meaning there may never be an easy field application of RNA biomarkers available to producers. Still, there may be a future for RNA biomarker use with in vitro fertilization (IVF) [87] or diagnostic tests of individuals with idiopathic infertility.

Genome

As it has been shown that RNA and proteins in spermatozoa and seminal plasma can be correlated with successful fertilization events and embryo development, it is likely the genome itself could aid in earlier prediction of higher fertility males. High-throughput genotyping with comparison to fertility records may be able to establish predictive fertility models. The current method for this is genome-wide association studies (GWAS), which are used to identify specific genomic regions associated with a trait. These studies scan single nucleotide polymorphism (SNP) markers for genetic variations, locating regions on chromosomes that harbor associated genes. Functional candidate genes are then identified by using the closest genes mapped around the candidate marker.

Specific phenotypic traits, related to the testes (scrotal circumference) and spermatozoa (percent normal sperm), have been targeted as indicative of reproductive efficiency [88, 89]. Ideally, identification of genetic markers linked to phenotypic traits will allow for the selection of these traits. Multiple regions on the X chromosome have been associated with testis development and sperm morphology [90], where two regions linked to scrotal circumference were located at 69–77 and 81–92 Mb, and polymorphisms that were associated with percent normal sperm were located between 40 and 55 Mb. On Bovine Chromosome 9 (BTA9), candidate regions identified through GWAS in relation to scrotal circumference and sperm motility have known genetic correlations to other valuable production traits, including conformation, daughter pregnancy rate, interval to first estrus after calving, and body weight gain [91]. With some fertility traits, such as sperm motility [92] and sperm production [93], the distribution of SNP effects indicates that they likely come from polygenic rather than simple inheritance. A comprehensive genomic analysis using dairy bulls identified eight genomic regions on six chromosomes associated with sire fertility, many of these harboring genes with known roles in sperm maturation, motility, and fertilization [94].

The use of genome-guided selection by the dairy industry has led to increased daughter pregnancy rate, productive life, and decreased milk somatic cell score, all of which are considered to have relatively low heritability [35]. In dairy bulls, SNP markers linked to sire conception rate have shown promise in predicting bull fertility [95–97]. Largely, GWAS have focused on testicular traits for beef breeds and spermatic traits for dairy breeds, though this appears to be more industry preference than breed relevance.

The GWAS may be able to help identify specific causes of idiopathic subfertility when genomic variation leads to a clear phenotypic effect. The discovery of a genomic region on BTA19 related to male reproductive performance revealed a common segment in 40 bulls with idiopathic subfertility [98]. A causative loss-of-function mutation had occurred in *TMEM95*, which encodes a protein found on the acrosomal membrane of the sperm head that is lost after acrosome reaction [99]. In mice, spermatozoa lacking the TMEM95 protein were morphologically normal with normal motility and are capable of both penetrating the zona pellucida and binding to the oolemma but were unable to fuse with the egg membrane and could not achieve fertilization without intracytoplasmic sperm injection to bypass the requirement of gamete fusion [100].

Along with identifying genes and associated traits, the genome may serve as a way to evaluate inbreeding levels [101], which have been shown to have a negative impact on sperm quality and field fertility [102]. Whole-genome homozygosity mapping, identifying runs of homozygosity, found eight segments to be significantly associated with sire conception rate [103]; most of these segments containing genes are thought to have important roles in testis development, spermatogenesis, or sperm function.

Metabolites

Another "omics" field showing potential for use in predicting male fertility is metabolomics; metabolites being the small molecules that result from metabolic reactions. As they are the products of important biochemical pathways, metabolites have the potential to give major insight into cellular function.

Many of the most abundant metabolites in bovine seminal plasma are involved in energy metabolism, but only a few studies correlating metabolites with bull fertility have been reported [104–108]. In the first study to identify fertility-associated metabolites in the bull, four metabolites in seminal plasma and four metabolites in blood serum showed significant differences between high and low fertility bulls [104]. In another study, seven metabolites were discovered with statistically significant differences between high and low fertility males [105]. Of these, fructose, 4-ketoglucose, and erythronic acid were more abundant in seminal plasma from high fertility bulls, while 2-oxoglutaric acid, phosphoric acid, D-mannitol, and dulcitol were more abundant in the seminal plasma of low fertility bulls.

Likewise in the spermatozoa of bulls, 22 distinct metabolites were identified, with 5 showing statistically significant differential expression between high and low fertility bulls [106]. The GABA, carabamate, benzoic acid, and lactic acid were found to be more abundant in the sperm of high fertility bulls, while palmitic acid was more abundant in low fertility bulls. The increased amount of lactic acid in the spermatozoa

of more fertile bulls could suggest that high fertility bulls are utilizing glycolysis more efficiently. Bull spermatozoa are able to utilize both glycolysis and oxidative phosphorylation (OXPHOS) as energy production pathways [109], and if glycolysis is utilized preferentially by the spermatozoa of high fertility males, metabolites associated with this pathway, such as lactic acid, may be able to be used to evaluate which energy production method is being favored and therefore fertility.

Figure 2A depicts the classes of 53 metabolites, more abundant in high or low fertility bulls, identified in spermatozoa [106–108], seminal plasma [104, 105, 107], and blood serum [104]. In Figure 2B, the classes of these same 53 metabolites are shown as they relate to fertility status. This suggests that certain types of metabolites will be more easily detected in spermatozoa or seminal plasma than in blood serum. From these identified metabolites, "nucleosides, nucleotides, and analogs" were only found in spermatozoa and were mostly associated with low fertility (Figure 2). The high percentage of "carbohydrates and carbohydrate conjugates" identified in seminal plasma as opposed to spermatozoa can be explained as providing necessary extracellular support for the energy production pathways of spermatozoa [110]. While the large percentage of fertility-associated "carbohydrates and carbohydrate conjugates" identified in low fertility bulls would seem to imply more support for energy production, it is mostly due to the small number of "amino acids, peptides, and analogs" and "other organic acids and derivatives" correlated to low fertility in these samples (see Supplemental Table S2 for a full list of metabolites). Many more fertility-associated "amino acids, peptides, and analogs" and "organic acids and derivatives" were identified in high fertility bulls than low fertility bulls (Figure 2B), which may simply show the more active energy metabolism of spermatozoa from high fertility bulls, as amino acids are involved in the regulation of metabolic activity and organic acids are produced by the breakdown of amino acids.

Some of the most noted metabolites in sperm biology are the reactive oxygen species (ROS). A by-product of the OXPHOS-mediated ATP production pathway, ROS play a role in the physiological maturation of spermatozoa, driving the tyrosine phosphorylation events necessary for capacitation [111]. The paradox of ROS is that while a certain level is necessary for proper function, the continued, unchecked generation of ROS eventually overwhelms the cells, triggering apoptosis [112]. In that respect, ROS are more often mentioned for their negative effects, including reduced motility and membrane integrity and increased DNA fragmentation [25]. Furthermore, nonviable or poor-quality sperm generate the most ROS [113]; so whether it is high levels of ROS contributing to poor sperm quality or poor-quality sperm producing more ROS, this may be a way to choose better samples for use.

Metabolomic biomarkers show the potential for future application in fertility assessments of bulls. This is undoubtedly the most promising biomarker family for use in a field setting. Metabolites are chemically reactive, which could in theory allow for the development of a relatively simple assay. However, metabolite biomarkers will be less a test of bull fertility and instead be more of a reflection of sample fertility. While other "omics" fields may be more specific in identifying the molecular factors responsible for low or high fertility, metabolites can be used to assess the endpoint of sperm function itself, which is perhaps more valuable, as fertility

status is not a fixed attribute and may change with animal age or season.

Other potential biomarkers

Though the methods already discussed in this paper appear to be the leading areas for developing biomarkers of fertility, others have shown potential as well. DNA methylation, a known mode of epigenetic inheritance, can be correlated with infertility. In a study comparing the DNA methylation levels of spermatozoa from bulls of differing fertility status, differentially methylated regions were identified on genes with functional roles in spermatogenesis, fertilization, and embryo development [114]. A recent human study concluded there was sufficient separation between fertile versus infertile patients, in a genome-wide analysis of DNA methylation, for diagnostic use [115]. Findings such as these create an important link between 'omics studies and actual diagnostic application, which opens up exciting possibilities should this same principle be true for DNA methylation in bulls.

Though technically a subset of metabolomics, the study of cellular lipids is emerging as its own 'omics field. The lipidomic profile of spermatozoa and seminal plasma may also provide insight into the fertility status of males. A recent human study has shown four fatty acids (palmitic acid, behenic acid, oleic acid, and DHA) to be biomarkers of semen quality, with stearic acid and DHA correlated to sperm motility [116]. While the lipidomic profile of human spermatozoa differs from that of the bull, with bull spermatozoa lower in cholesterol [117] and having a higher ratio of polyunsaturated fatty acids to saturated fatty acids [118], lipids could give insight into the quality of bull semen as well. Fatty acid composition has also been suggested as a possible explanation for the variability observed among individuals to withstand cryopreservation, with differences being detected in 'good' and 'poor' freezers [119]. Variations in the fatty acid and cholesterol compositions of spermatozoa and seminal plasma have also been reported in relation to the seasonal effect on semen quality [120]. These studies suggest that lipid composition may be more useful as a quality assessment, or for determining the freezability of a bull at a particular point in time, than as predictive biomarkers. Lipid profiles are perhaps one of the most encouraging areas of assessing fertility as it is the only one that could be supported or altered either through additives in conventional semen extenders [121] or through feed supplementation [122].

Future directions

It is highly unlikely that a single biomarker will ever be able to perfectly predict fertility, as fertility is too complex and multifactorial to be so easily defined. A multifaceted approach using many identified biomarkers would be better suited to assessing male fertility. While it may seem that there is a surplus of emerging technologies attempting to answer the same fertility question, there are potential uses (and limitations) for all of them in the future of livestock breeding.

Different families of biomarkers lend themselves to different applications. Genomic screening of immature bulls will allow for the early removal of bulls with a high likelihood of infertility. This would be beneficial to producers as it reduces financial loss both from raising an infertile bull and from low herd productivity. While the genome is fixed and
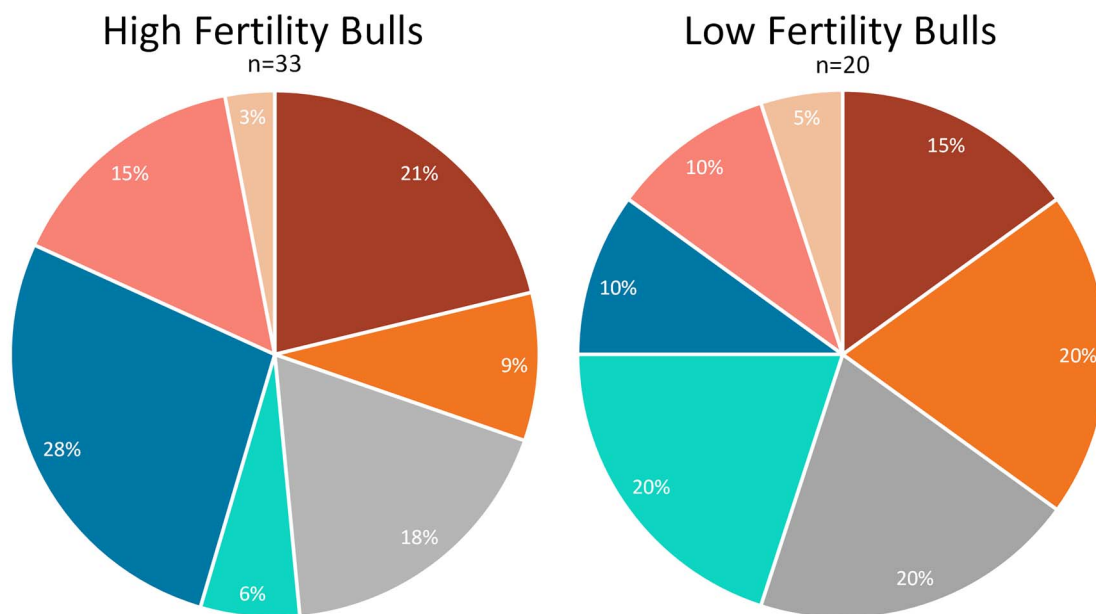
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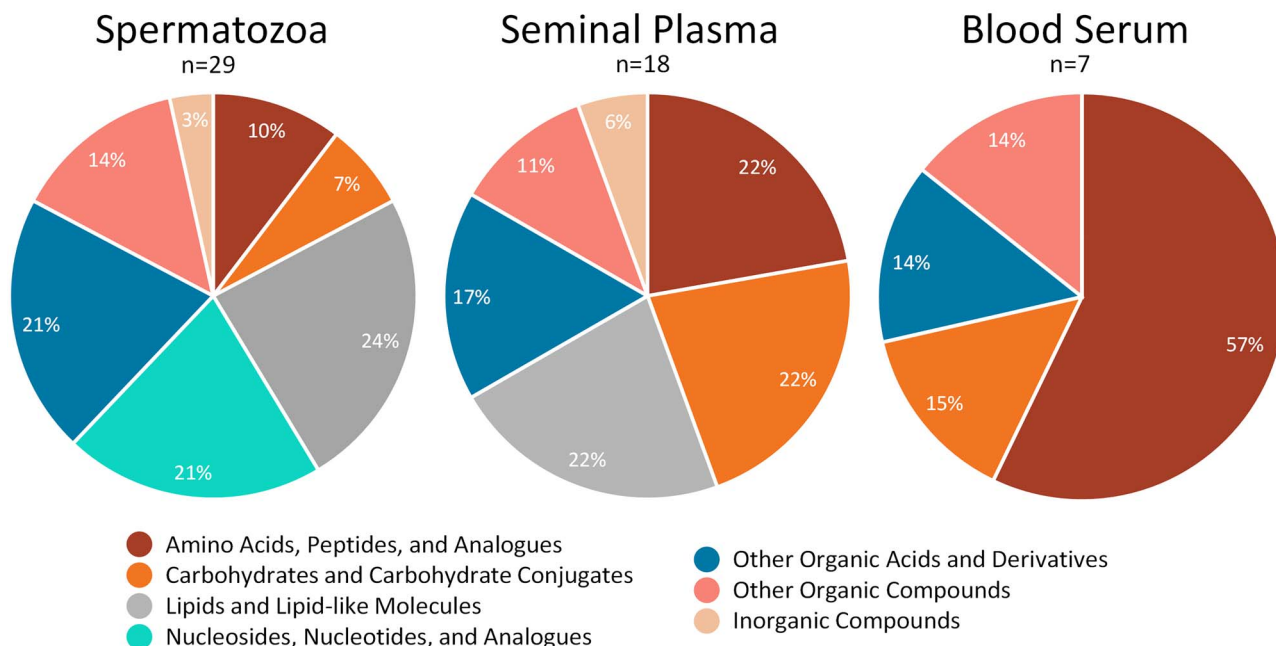
Figure 2. Metabolite biomarkers of fertility could come from a variety of different classes. (A) A breakdown of the classes of 53 metabolites correlated with fertility status that were identified in spermatozoa [106–108], seminal plasma [104, 105, 107], or blood serum [104]. (B) The identified metabolite classes shown as more abundant in high or low fertility bulls (for more details, see Supplemental Table S2).

can be evaluated in immature bulls, a bull would need to reach maturity before sperm and seminal plasma biomarkers are applicable. Proteomic markers could be used to evaluate certain sperm traits, such as the ability of spermatozoa to capacitate and bind to the zona pellucida, while transcriptomic markers could give insight into the spermatozoon's capability to support embryo development. These biomarkers may always need to be evaluated in a laboratory setting, which would not be practical nor cost-effective for all producers, but their identification creates new possibilities for use in certain circumstances, such as with IVF or diagnosing idiopathic infertility. The metabolome of bull sperm and seminal plasma

can be more variable in the individual than other biomarker families; however, assays developed for metabolic markers of fertility have the potential to be quicker and easier to use in the field. Such assays could be used to identify periods of subfertility due to environmental factors, such as heat stress, or in conjuncture with yearly BSEs for re-evaluating fertility status with aging.

As various methods of sperm processing and storage can cause damage to the spermatozoa, and/or leave spermatozoa exposed to undiluted seminal plasma for longer, different molecular components may be found to be beneficial or detrimental in different situations. Protein composition could

be useful as an indicator of freezability, which is known to vary independently of fertility status, as some proteins have been found to be more abundant in the seminal plasma of bulls with high freezability [55]. Similarly, certain seminal plasma metabolites have been shown to be associated with pregnancy rates following insemination with sex-sorted spermatozoa [123], suggesting metabolomics may be capable of identifying samples that are more tolerant of the sex-sorting process. Importantly, different species may require the development of different biomarker assays as the highly abundant transcripts found in *Bos indicus* spermatozoa appear to be different from those most common in *Bos taurus* spermatozoa [124], all further proving that there is no simple answer to the question of fertility and that no single biomarker is applicable to all situations.

While many molecular factors have shown potential as biomarkers of fertility, there is always a lag between the discovery of new knowledge and industry adoption. Further research needs to be done to determine the expression profiles that define fertile or subfertile males and to confirm the use of those biomarkers for diagnostic and predictive purposes before they can become clinically applicable.

Conclusion

Genetic material is unarguably the most important contribution of the spermatozoon to the embryo, but it is not its only contribution of value. Molecular components of spermatozoa, such as proteins, RNAs, and metabolites, likely have more of a role in fertilization and embryo development than previously thought. These promising molecular components have reported correlations with fertility, with the potential to create new methods of evaluating bull fertility. Due to the complexity of fertilizing events and the vast number of factors involved, usable biomarkers will most likely come in the form of identifiers of poor fertility, as with an absence of PEPB4 [58] or TMEM95 [98] proteins. Such markers could be used to speed up subfertility diagnosis—allowing for faster intervention in breeding programs with low pregnancy rates, or screening to select individuals with a higher likelihood of fertility—improving both management and economic outcomes for producers.

Supplementary material

Supplementary material is available at BIOLRE online.

Authors' contributions

E.K.K. and Z.G. conceived of the review article. E.K.K. wrote the manuscript and prepared the figures and supplemental tables. A.S., A.J.G., C.P.S., R.J.A., and Z.G. revised the manuscript.

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Figure 1B was created using images from BioRender.com.

Conflict of Interest

The authors have declared that no conflict of interest exists. The funders had no role in the conceptualization or writing of this review.

References

1. Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC, Memili E. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst Biol* 2008; **2**:19.
2. Bissonnette N, Lévesque-Sergerie JP, Thibault C, Boissonneault G. Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. *Reproduction* 2009; **138**:65.
3. Feugang JM, Rodriguez-Osorio N, Kaya A, Wang H, Page G, Ostermeier GC, Topper EK, Memili E. Transcriptome analysis of bull spermatozoa: implications for male fertility. *Reprod Biomed Online* 2010; **21**:312–324.
4. Foroutan A, Fitzsimmons C, Mandal R, Piri-Moghadam H, Zheng J, Guo AC, Li C, Guan LL, Wishart DS. The bovine metabolome, *Metabolites* 2020; **10**:233.
5. Morrell JM. Heat stress and bull fertility. *Theriogenology* 2020; **153**:62–67.
6. Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, Bollwein H. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. *Theriogenology* 2016; **86**: 562–571.
7. Buffone MG, Ijiri TW, Cao W, Merdiushev T, Aghajanian HK, Gerton GL. Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. *Mol Reprod Dev* 2012; **79**:4–18.
8. Navara CS, First NL, Schatten G. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis, and nuclear transfer: the role of the sperm aster. *Dev Biol* 1994; **162**: 29–40.
9. Immler S. The sperm factor: paternal impact beyond genes. *Heredity* 2018; **121**:239–247.
10. Sullivan R, Mieusset R. The human epididymis: its function in sperm maturation. *Hum Reprod Update* 2016; **22**:574–587.
11. Barth AD. Case-based studies of infertility in bulls. In: *45th Annual Conference. American Association of Bovine Practitioners*. Montreal, Canada; 2012.
12. Koziol JH, Armstrong CL. *Society for Theriogenology Manual for Breeding Soundness Examination of Bulls*. Society for Theriogenology; 2018.
13. Chenoweth PJ, Spitzer JC, Hopkins FM. A new bull breeding soundness evaluation form. In: *Proceedings for the Annual Meeting of the Society for Theriogenology*. Montgomery, AL, USA; 1992.
14. Hopkins FM, Spitzer JC. The new Society for Theriogenology Breeding Soundness Evaluation System. *Vet Clin North Am Food Anim* 1997; **13**:283–293.
15. Fordyce G, Entwistle K, Norman S, Perry V, Gardiner B, Fordyce P. Standardising bull breeding soundness evaluations and reporting in Australia. *Theriogenology* 2006; **66**:1140–1148.
16. Penny CD. The new BCVA bull pre-breeding examination certificate. *Cattle Practice* 2009; **17**:202–207.
17. Barth AD. *Bull Breeding Soundness*, 3rd ed. Western Canadian Association of Bovine Practitioners; 2013.
18. Irons PC, Nöthling JO, Bertschinger HJ. Bull breeding soundness evaluation in southern Africa. *Theriogenology* 2007; **68**: 842–847.
19. Barth AD. Review: the use of bull breeding soundness evaluation to identify subfertile and infertile bulls. *Animal* 2018; **12**:s158–s164.
20. Kastelic J, Thundathil J. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim* 2008; **43**:368–373.
21. De Jonge C. Attributes of fertile spermatozoa: an update. *J Androl* 1999; **20**:463–473.
22. DeJarnette JM. The effect of semen quality on reproductive efficiency. *Vet Clin Food Anim* 2005; **21**:409–418.

23. Correa JR, Pace MM, Zavos PM. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology* 1997; **48**:721–731.

24. Kasimanickam R, Nebel RL, Peeler ID, Silvia WL, Wolf KT, McAllister AJ, Cassell BG. Breed differences in competitive indices of Holstein and Jersey bulls and their association with sperm DNA fragmentation index and plasma membrane integrity. *Theriogenology* 2006; **66**:1307–1315.

25. Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL, Cassell BG. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology* 2007; **67**: 1004–1012.

26. Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biol Reprod* 1987; **36**:915–925.

27. Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *J Reprod Fertil* 1980; **59**:463–468.

28. Roche JF, Bolandl MP, McGeady TA. Reproductive wastage following artificial insemination of heifers. *Vet Rec* 1981; **109**: 401–404.

29. Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Mol Reprod Dev* 1994; **37**:48–53.

30. Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD. Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. *Biol Reprod* 2001; **64**:1624–1632.

31. Tesarik J. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. *Hum Reprod* 2002; **17**:184–189.

32. Skerget S, Rosenow MA, Petritis K, Karr TL. Sperm proteome maturation in the mouse epididymis. *PLOS ONE* 2015; **10**:e0140650.

33. Krawetz SA, Kruger A, Lalancette C, Tagett R, Anton E, Draghici S, Diamond MP. A survey of small RNAs in human sperm. *Hum Reprod* 2011; **26**:3401–3412.

34. Courant F, Antignac JP, Monteau F, Le Bizec B. Metabolomics as a potential new approach for investigating human reproductive disorders. *J. Proteome Res.* 2013; **12**:2914–2920.

35. García-Ruiz A, Cole JB, VanRaden PM, Wiggans GR, Ruiz-López FJ, van Tassell CP. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. *Proc Natl Acad Sci* 2016; **113**:E3995.

36. Leahy T, Rickard JP, Pini T, Gadella BM, Graaf SP. Quantitative proteomic analysis of seminal plasma, sperm membrane proteins, and seminal extracellular vesicles suggests vesicular mechanisms aid in the removal and addition of proteins to the ram sperm membrane. *Proteomics* 2020; **20**:1900289.

37. Griffin RA, Swegen A, Baker M, Aitken RJ, Skerrett-Byrne DA, Silva Rodriguez A, Martín-Cano FE, Nixon B, Peña FJ, Delehedde M, Sergeant N, Gibb Z. Mass spectrometry reveals distinct proteomic profiles in high- and low-quality stallion spermatozoa. *Reproduction* 2020; **160**:695–707.

38. Mills KM, Aryal UK, Sobreira T, Minton AM, Casey T, Stewart KR. Shotgun proteome analysis of seminal plasma differentiate boars by reproductive performance. *Theriogenology* 2020; **157**: 130–139.

39. Frenette G, Sullivan R. Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. *Mol Reprod Dev* 2001; **59**:115–121.

40. Bellin ME, Hawkins HE, Ax RL. Fertility of range beef bulls grouped according to presence or absence of heparin-binding proteins in sperm membranes and seminal fluid. *J Anim Sci* 1994; **72**:2441–2448.

41. Bellin ME, Hawkins HE, Oyarzo JN, Vanderboom RJ, Ax RL. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *J Anim Sci* 1996; **74**:173–182.

42. Bellin ME, Oyarzo JN, Hawkins HE, Zhang H, Smith RG, Forrest DW, Sprott LR, Ax RL. Fertility-associated antigen on bull sperm indicates fertility potential. *J Anim Sci* 1998; **76**:2032–2039.

43. McCauley TC, Zhang H, Bellin ME, Ax RL. Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. *Mol Reprod Dev* 1999; **54**:145–153.

44. Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biol Reprod* 1998; **58**:826–833.

45. Moura AA. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *J Androl* 2006; **27**:201–211.

46. Parent S, Lefivre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. *Mol Reprod Dev* 1999; **52**:57–65.

47. Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. *Hum Reprod Update* 2014; **20**:40–62.

48. Berube B, Sullivan R. Inhibition of in vivo fertilization by active immunization of male hamsters against a 26-kDa sperm glycoprotein. 1994; **51**:1255–1263.

49. Boué F, Bérubé B, de Lamirande E, Gagnon C, Sullivan R. Human sperm-zona pellucida interaction is inhibited by an antiserum against a hamster sperm protein. *Biol Reprod* 1994; **51**:577–587.

50. Nauc V, Manjunath P. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/−A2, BSP-A3, and BSP-30-Kilodaltons), and their quantification in seminal plasma and sperm. *Biol Reprod* 2000; **63**:1058–1066.

51. Roncoletta M, Morani ESC, Esper CR, Barnabe VH, Franceschini PH. Fertility-associated proteins in Nelore bull sperm membranes. *Anim Reprod Sci* 2006; **91**:77–87.

52. Manjunath P, Nauc V, Bergeron A, Ménard M. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. *Biol Reprod* 2002; **67**:1250–1258.

53. Pereira GR, de Lazari FL, Dalberto PF, Bizarro CV, Sontag ER, Koetz Junior C, Menegassi SRO, Barcellos JOJ, Bustamante-Filho IC. Effect of scrotal insulation on sperm quality and seminal plasma proteome of Brangus bulls. *Theriogenology* 2020; **144**: 194–203.

54. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* 2006; **75**:501–507.

55. Jobim MIM, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. *Theriogenology* 2004; **61**: 255–266.

56. D'Amours O, Frenette G, Fortier M, Leclerc P, Sullivan R. Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. *Reproduction* 2010; **139**:545.

57. Soggiu A, Piras C, Hussein HA, de Canio M, Gaviraghi A, Galli A, Urbani A, Bonizzi L, Roncada P. Unravelling the bull fertility proteome. *Mol Biosyst* 2013; **9**:1188–1195.

58. Somashekar L, Selvaraju S, Parthipan S, Patil SK, Binsila BK, Venkataswamy MM, Karthik Bhat S, Ravindra JP. Comparative sperm protein profiling in bulls differing in fertility and identification of phosphatidylethanolamine-binding protein 4, a potential fertility marker. *Andrology* 2017; **5**:1032–1051.

59. Kasimanickam RK, Kasimanickam VR, Arangasamy A, Kastelic JP. Sperm and seminal plasma proteomics of high- versus low-fertility Holstein bulls. *Theriogenology* 2019; **126**: 41–48.

60. Viana AGA, Martins AMA, Pontes AH, Fontes W, Castro MS, Ricart CAO, Sousa MV, Kaya A, Topper E, Memili E, Moura AA. Proteomic landscape of seminal plasma associated with dairy bull fertility. *Sci Rep* 2018; **8**:16323.

61. Willforss J, Morrell JM, Resjö S, Hallap T, Padrik P, Siino V, de Koning DJ, Andreasson E, Levander F, Humblot P. Stable bull fertility protein markers in seminal plasma. *J Proteomics* 2021; **236**:104135.

62. Westfalewicz B, Dietrich MA, Ciereszko A. Impact of cryopreservation on bull (Bos taurus) semen proteome. *J Anim Sci* 2015; **93**: 5240–5253.

63. Park Y-J, Kwon WS, Oh SA, Pang MG. Fertility-related proteomic profiling bull spermatozoa separated by percoll. *J Proteome Res* 2012; **11**:4162–4168.

64. Park Y-J, Pang WK, Ryu DY, Song WH, Rahman MS, Pang MG. Optimized combination of multiple biomarkers to improve diagnostic accuracy in male fertility. *Theriogenology* 2019; **139**: 106–112.

65. Amaral PP, Dinger ME, Mercer TR, Mattick JS. The eukaryotic genome as an RNA machine. *Science* 2008; **319**:1787–1789.

66. Miller D, Ostermeier GC, Krawetz SA. The controversy, potential and roles of spermatozoal RNA. *Trends Mol Med* 2005; **11**: 156–163.

67. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Delivering spermatozoan RNA to the oocyte. *Nature* 2004; **429**:154–154.

68. Guo L, Chao SB, Xiao L, Wang ZB, Meng TG, Li YY, Han ZM, Ouyang YC, Hou Y, Sun QY, Ou XH. Sperm-carried RNAs play critical roles in mouse embryonic development. *Oncotarget* 2017; **8**:67394–67405.

69. Lalancette C, Thibault C, Bachand I, Caron N, Bissonnette N. Transcriptome analysis of bull semen with extreme nonreturn rate: use of suppression-subtractive hybridization to identify functional markers for fertility. *Biol Reprod* 2008; **78**: 618–635.

70. Selvaraju S, Parthipan S, Somashekar L, Kolte AP, Krishnan Binsila B, Arangasamy A, Ravindra JP. Occurrence and functional significance of the transcriptome in bovine (*Bos taurus*) spermatozoa. *Sci Rep* 2017; **7**:42392.

71. Nixon B, Stanger SJ, Mihalas BP, Reilly JN, Anderson AL, Tyagi S, Holt JE, McLaughlin EA. The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. *Biol Reprod* 2015; **93**:91.

72. Conine CC, Sun F, Song L, Rivera-Pérez JA, Rando OJ. Small RNAs gained during epididymal transit of sperm are essential for embryonic development in mice. *Dev Cell* 2018; **46**:470–480.e3.

73. Fagerlind M, Stålhammar H, Olsson B, Klinga-Levan K. Expression of miRNAs in bull spermatozoa correlates with fertility rates. *Reprod Dom Anim* 2015; **50**:587–594.

74. Capra E, Turri F, Lazzari B, Cremonesi P, Gliozzi TM, Fojadelli I, Stella A, Pizzi F. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between high- and low-motile sperm populations. *BMC Genom* 2017; **18**:1, 14–12.

75. Shangguan A, Zhou H, Sun W, Ding R, Li X, Liu J, Zhou Y, Chen X, Ding F, Yang L, Zhang S. Cryopreservation induces alterations of miRNA and mRNA fragment profiles of bull sperm. *Front Genet* 2020; **11**:419.

76. Wang M, Gao Y, Qu P, Qing S, Qiao F, Zhang Y, Mager J, Wang Y. Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. *Sci Rep* 2017; **7**:13403.

77. Sellem E, Marthey S, Rau A, Jouneau L, Bonnet A, Perrier JP, Fritz S, le Danvic C, Boussaha M, Kiefer H, Jammes H, Schibler L. A comprehensive overview of bull sperm-borne small non-coding RNAs and their diversity across breeds. *Epigenetics Chromatin* 2020; **13**:19.

78. Felton-Taylor J, Prosser KA, Hernandez-Medrano JH, Gentili S, Copping KJ, Macrossan PE, Perry VEA. Effect of breed, age, season and region on sperm morphology in 11,387 bulls submitted to breeding soundness evaluation in Australia. *Theriogenology* 2020; **142**:1–7.

79. Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, Farinelli L, Miska E, Mansuy IM. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat Neurosci* 2014; **17**:667.

80. Rodgers AB, Morgan CP, Leu NA, Bale TL. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc Natl Acad Sci* 2015; **112**: 13699–13704.

81. Claycombe-Larson KG, Bundy AN, Roemmich JN. Paternal high-fat diet and exercise regulate sperm miRNA and histone methylation to modify placental inflammation, nutrient transporter mRNA expression and fetal weight in a sex-dependent manner. *J Nutr Biochem* 2020; **81**:108373.

82. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, Feng GH, Peng H, Zhang X, Zhang Y, Qian J, Duan E *et al.* Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science* 2016; **351**:397–400.

83. Trigg NA, Eamens AL, Nixon B. The contribution of epididymosomes to the sperm small RNA profile. *Reproduction* 2019; **157**:R209–R223.

84. Sharma U. Paternal contributions to offspring health: role of sperm small RNAs in intergenerational transmission of epigenetic information. *Front Cell Dev Biol* 2019; **7**: 215.

85. Sharma U, Sun F, Conine CC, Reichholf B, Kukreja S, Herzog VA, Ameres SL, Rando OJ. Small RNAs are trafficked from the epididymis to developing mammalian sperm. *Dev Cell* 2018; **46**:481–494.

86. Gilbert I, Bissonnette N, Boissonneault G, Vallée M, Robert C. A molecular analysis of the population of mRNA in bovine spermatozoa. *Reproduction* 2007; **133**:1073–1086.

87. Hua M, Liu W, Chen Y, Zhang F, Xu B, Liu S, Chen G, Shi H, Wu L. Identification of small non-coding RNAs as sperm quality biomarkers for in vitro fertilization. *Cell Discovery* 2019; **5**:20.

88. Waldner CL, Kennedy RI, Palmer CW. A description of the findings from bull breeding soundness evaluations and their association with pregnancy outcomes in a study of western Canadian beef herds. *Theriogenology* 2010; **74**:871–883.

89. Fitzpatrick LA, Fordyce G, McGowan MR, Bertram JD, Doogan VJ, de Faveri J, Miller RG, Holroyd RG. Bull selection and use in northern Australia: part 2. Semen traits. *Anim Reprod Sci* 2002; **71**:39–49.

90. Fortes MRS, Reverter A, Kelly M, McCulloch R, Lehnert SA. Genome-wide association study for inhibin, luteinizing hormone, insulin-like growth factor 1, testicular size and semen traits in bovine species. *Andrology* 2013; **1**:644–650.

91. Sweett H, Fonseca PAS, Suárez-Vega A, Livernois A, Miglior F, Cánovas A. Genome-wide association study to identify genomic regions and positional candidate genes associated with male fertility in beef cattle. *Sci Rep* 2020; **10**:20102.

92. Hering DM, Olenski K, Kaminski S. Genome-wide association study for poor sperm motility in Holstein-Friesian bulls. *Anim Reprod Sci* 2014; **146**:89–97.

93. Sang L, du QZ, Yang WC, Tang KQ, Yu JN, Hua GH, Zhang XX, Yang LG. Polymorphisms in follicle stimulation hormone receptor, inhibin alpha, inhibin bata A, and prolactin genes, and their association with sperm quality in Chinese Holstein bulls. *Anim Reprod Sci* 2011; **126**:151–156.

94. Han Y, Peñagaricano F. Unravelling the genomic architecture of bull fertility in Holstein cattle. *BMC Genet* 2016; **17**:1.

95. Rezende FM, Nani JP, Peñagaricano F. Genomic prediction of bull fertility in US Jersey dairy cattle. *J Dairy Sci* 2019; **102**: 3230–3240.

96. Nani JP, Rezende FM, Peñagaricano F. Predicting male fertility in dairy cattle using markers with large effect and functional annotation data. *BMC Genom* 2019; **20**:258.

97. Pacheco HA, Rezende FM, Peñagaricano F. Gene mapping and genomic prediction of bull fertility using sex chromosome markers. *J Dairy Sci* 2020; **103**:3304–3311.

98. Pausch H, Kölle S, Wurmser C, Schwarzenbacher H, Emmerling R, Jansen S, Trottmann M, Fuerst C, Götz KU, Fries R. A nonsense mutation in TMEM95 encoding a nondescript transmembrane protein causes idiopathic male subfertility in cattle. *PLoS Genet* 2014; **10**:e1004044.

99. Fernandez-Fuertes B, Laguna-Barraza R, Fernandez-Gonzalez R, Gutierrez-Adan A, Blanco-Fernandez A, O'Doherty AM, di Fenza M, Kelly AK, Kölle S, Lonergan P. Subfertility in bulls carrying a nonsense mutation in transmembrane protein 95 is due to failure to interact with the oocyte vestments†. *Biol Reprod* 2017; **97**: 50–60.

100. Lamas-Toranzo I, Hamze JG, Bianchi E, Fernández-Fuertes B, Pérez-Cerezales S, Laguna-Barraza R, Fernández-González R, Lonergan P, Gutiérrez-Adán A, Wright GJ, Jiménez-Movilla M, Bermejo-Álvarez P. TMEM95 is a sperm membrane protein essential for mammalian fertilization. *elife* 2020; **9**:e53913.

101. Howard JT, Pryce JE, Baes C, Maltecca C. Invited review: inbreeding in the genomics era: inbreeding, inbreeding depression, and management of genomic variability. *J Dairy Sci* 2017; **100**: 6009–6024.

102. Dorado J, Cid RM, Molina A, Hidalgo M, Ariza J, Moreno-Millán M, Demyda-Peyrás S. Effect of inbreeding depression on bull sperm quality and field fertility. *Reprod Fertil Dev* 2017; **29**:712.

103. Nani JP, Peñagaricano F. Whole-genome homozygosity mapping reveals candidate regions affecting bull fertility in US Holstein cattle. *BMC Genom* 2020; **21**:338.

104. Kumar A, Kroetsch T, Blondin P, Anzar M. Fertility-associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. *Mol Reprod Dev* 2015; **82**: 123–131.

105. Velho ALC, Menezes E, Dinh T, Kaya A, Topper E, Moura AA, Memili E. Metabolomic markers of fertility in bull seminal plasma. *PLOS ONE* 2018; **13**:e0195279.

106. Menezes EB, Velho ALC, Santos F, Dinh T, Kaya A, Topper E, Moura AA, Memili E. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genom* 2019; **20**:714.

107. Longobardi V, Kosior MA, Pagano N, Fatone G, Staropoli A, Vassetti A, Vinale F, Campanile G, Gasparrini B. Changes in bull semen metabolome in relation to cryopreservation and fertility. *Animals* 2020; **10**:1065.

108. Saraf KK, Kumaresan A, Dasgupta M, Karthikkeyan G, Prasad TSK, Modi PK, Ramesha K, Jeyakumar S, Manimaran A. Metabolomic fingerprinting of bull spermatozoa for identification of fertility signature metabolites. *Mol Reprod Dev* 2020; **87**: 692–703.

109. Storey BT. Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* 2008; **52**:427–437.

110. Mann T. Studies on the metabolism of semen: 3. Fructose as a normal constituent of seminal plasma. Site of formation and function of fructose in semen. *Biochem J* 1946; **40**:481–491.

111. Aitken RJ, Nixon B. Sperm capacitation: a distant landscape glimpsed but unexplored. *Mol Hum Reprod* 2013; **19**:785–793.

112. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol Reprod* 2012; **87**: 110–110.

113. Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine spermatozoa. *Am J Vet Res* 2001; **62**:508–515.

114. Kropp J, Carrillo JA, Namous H, Daniels A, Salih SM, Song J, Khatib H. Male fertility status is associated with DNA methylation signatures in sperm and transcriptomic profiles of bovine preimplantation embryos. *BMC Genom* 2017; **18**: 1–15.

115. Luján S, Caroppo E, Niederberger C, Arce JC, Sadler-Riggleman I, Beck D, Nilsson E, Skinner MK. Sperm DNA methylation epimutation biomarkers for male infertility and FSH therapeutic responsiveness. *Sci Rep* 2019; **9**:16786.

116. Zerbinati C *et al.* Fatty acids profiling reveals potential candidate markers of semen quality. *Andrology* 2016; **4**:1094–1101.

117. Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology* 1977; **14**:466–470.

118. Poulos A, Darin-Bennett A, White IG. The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. *Comp Biochem Physiol B* 1973; **46**:541–549.

119. Evans HC, Dinh TTN, Ugur MR, Hitit M, Sajeev D, Kaya A, Topper E, Nicodemus MC, Smith GD, Memili E. Lipidomic markers of sperm cryotolerance in cattle. *Sci Rep* 2020; **10**: 20192.

120. Argov-Argaman N, Mahgrefthe K, Zeron Y, Roth Z. Season-induced variation in lipid composition is associated with semen quality in Holstein bulls. *Reproduction* 2013; **145**:479–489.

121. Kaka A, Haron W, Yusoff R, Yimer N, Khumran AM, Sarsaifi K, Behan AA, Kaka U, Memon AA, Ebrahimi M. Effect of docosahexanoic acid on quality of frozen–thawed bull semen in BioXcell extender. *Reprod Fertil Dev* 2017; **29**:490–495.

122. Gholami H, Chamani M, Towhidi A, Fazeli MH. Effect of feeding a docosahexaenoic acid-enriched nutriceutical on the quality of fresh and frozen-thawed semen in Holstein bulls. *Theriogenology* 2010; **74**:1548–1558.

123. Holden SA, Fernandez-Fuertes B, Murphy C, Whelan H, O'Gorman A, Brennan L, Butler ST, Lonergan P, Fair S. Relationship between in vitro sperm functional assessments, seminal plasma composition, and field fertility after AI with either non-sorted or sex-sorted bull semen. *Theriogenology* 2017; **87**: 221–228.

124. Raval NP, Shah TM, George LB, Joshi CG. Insight into bovine (*Bos indicus*) spermatozoal whole transcriptome profile. *Theriogenology* 2019; **129**:8–13.