What makes a fertile sperm? Unique molecular attributes of stallion fertility

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Abstract

Stallions experience lower per-cycle conception rates compared to other livestock species, largely because they are selected for breeding based on athletic prowess and not reproductive fitness. Mares are seasonal breeders, and pregnancies cannot be detected until 10–14 days post cover via transrectal ultrasonography. This means the detection of stallion fertility fluctuations is delayed by at least 2 weeks, which within the short breeding season employed by the thoroughbred horse breeding industry, can prove quite costly. For these reasons, there is increased demand for robust laboratory assays aimed at the accurate assessment of stallion fertility. This paper reviews our existing knowledge concerning the molecular mechanisms that underpin the functional competence of stallion spermatozoa, highlighting the relative importance of oxidative stress, DNA damage, sperm proteomics and RNA profile. We also consider the way in which fundamental improvements in our understanding of stallion sperm biology are informing the identification and development of possible biomarkers of fertility and thus avenues for the development of specific assays for fertility prediction.

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Introduction

The selection of horses for breeding is based on athletic performance and pedigree, rather than reproductive fitness. As a result, similar to humans, there is virtually no selection pressure for fertility (Parlevliet & Colenbrander 1999, Morris & Allen 2002, Novak *et al.* 2010, Gibb *et al.* 2014), and therefore, per-cycle conception (PCC) rates are generally lower than those observed for other domestic livestock species at around 60% (Nath *et al.* 2010).

The thoroughbred horse industry is unique from other horse breeding industries in that all registered foals must be produced via natural breeding and the use of assisted reproductive technologies is strictly forbidden. Furthermore, there are strict breeding seasons implemented by the industry in the northern and southern hemispheres. Foals born early in the season gain a chronologic and physiological advantage over late born foals in both the sales ring (Robbins & Kennedy 2001, Pagan *et al.* 2005) and at the race track (Oki *et al.* 1994, Gramm & Marksteiner 2010, Takahashi 2015), thus placing immense pressure on breeders to produce foals as close to the start of the season as possible.

A dependence on natural breeding means that popular stallions will breed up to four times a day. Any decrease in fertility resulting in a failed conception exacerbates the breeding pressure placed on stallions, as mares will have to be rebred on the following oestrous cycle. The ability to predict the existence of subfertility would allow

management personnel to better tailor the breeding regimens of individual stallions in order to maximise PCC rates. Although the samples cannot be used for insemination, semen from thoroughbred stallions is routinely collected for the purpose of performing semen assessments throughout the season. While conventional sperm assays, such as sperm concentration, motility and morphological assessments are a valuable first port-of-call for identifying subfertile stallions, they provide only moderate information on the functional competence of sperm cells (Petrunkina *et al.* 2007), and can be deleteriously affected by poor collection and handling techniques which result in a sample that is not representative of the original ejaculate. The use of more robust molecular assays which are not affected by post-ejaculatory handling would provide a more comprehensive understanding of the fertilising potential of the spermatozoa and a more reliable indication of any inter-ejaculation variation in fertility.

In this review, we provide an overview of recent developments in our understanding of molecular mechanisms that dictate sperm function in the horse that might potentially serve as clinically-useful indicators of a thoroughbred stallion's fertility. This includes aspects of sperm metabolism and its relationship with oxidative damage, the susceptibility of sperm DNA and proteins to the downstream effects of reactive oxygen species (ROS), the role of the sperm proteome in defining the fertilising potential of the spermatozoa and the emerging contributions of various RNA species to sperm function.

Sperm metabolism and oxidative damage

Like all cells, spermatozoa exist under aerobic conditions and, as a consequence, are faced with the oxygen paradox; oxygen is necessary for sustained life, but metabolites of oxidative processes, when present in high quantities, endanger the cell's survival (Baumber *et al.* 2000, Aitken *et al.* 2012b, Chandra *et al.* 2012). In comparison to human and mouse spermatozoa which predominantly use glycolysis to generate ATP, equine spermatozoa mainly employ oxidative phosphorylation (OXPHOS) for this purpose (Gibb *et al.* 2014). OXPHOS can generate approximately 36 ATP molecules in the metabolism of glucose compared to only two ATP molecules produced using glycolysis. Therefore, OXPHOS facilitates significantly faster motility, with stallion spermatozoa recording velocity parameters 60% higher than those of glycolytic human spermatozoa (Gibb *et al.* 2014). ROS are generated spontaneously by the cell as by-products during normal mitochondrial metabolism. These include the superoxide anion, nitric oxide and hydrogen peroxide, all of which are important signalling molecules in many cellular processes such as tyrosine phosphorylation during sperm capacitation (Dalle-Donne *et al.* 2003, Koppers *et al.* 2008, Aitken *et al.* 2010a).

The increased mitochondrial activity facilitated by OXPHOS results in a high rate of ROS generation. Although the sperm cell has a unique matrix of antioxidants which allow it to neutralise free radicals, this ability is lost over time once the antioxidant defences become overwhelmed (Gibb *et al.* 2016). Eventually the continued, unchecked production of these reactive molecules will lead to the perturbation of cellular functions (Baumber *et al.* 2000, Dalle-Donne *et al.* 2003, Aitken *et al.* 2010b), which can lead to motility loss and oxidative DNA damage, a phenomenon that increases the likelihood of embryo loss or birth defects in the offspring (Aitken *et al.* 2010b, Chandra *et al.* 2012). Oxidative stress is characterised as a state in which the rate of ROS generation exceeds the rate of its degradation within cells (Baumber *et al.* 2000). Once the cell has entered a state of oxidative stress, a cascade of detrimental events is initiated, commencing with loss of mitochondrial membrane potential and perturbation of the electron transport chain (ETC) and culminating in hydrogen abstraction from the polyunsaturated fatty acid (PUFA)-rich cell membrane and the generation of cytotoxic aldehydes (Koppers *et al.* 2008, Aitken *et al.* 2012a). Further downstream, oxidative stress may lead to the modification of both enzymatic and non-enzymatic antioxidants, leading to a loss of ability to neutralise free radicals by the sperm cell (Mohanty *et al.* 2016). Therefore, any minor increase in oxidative stress experienced by spermatozoa may proliferate rapidly and result in detrimental damage to sperm motility, viability and fertility (Aitken *et al.* 2012a, Aitken 2018).

The vulnerability of spermatozoa to oxidative attack stems from their ability to generate ROS, their lack of antioxidant protection and the abundance of substrates for oxidative attack including PUFA, proteins and DNA (Jones & Stewart 1979, Aitken *et al.* 2010a, Shiva *et al.* 2011). Indeed, oxidative stress is now considered to be a major contributing factor in virtually all disease processes, including infertility (Dalle-Donne *et al.* 2003).

The evolutionary pressures arising from the horse's social structure, behavioural breeding strategy and environment are likely to be the driving force for such a high reliance of stallion spermatozoa on OXPHOS. In the natural breeding system, horses typically form a harem where one stallion protects and breeds with several mares. Stallions have therefore evolved to be sensitive detectors of oestrus and will breed quickly and efficiently, close to the time of ovulation. Since mating and ovulation are naturally well synchronised in this species, evolutionary pressure to extend sperm survival appears to have been minimal. Furthermore, where sperm competition (i.e. postcopulatory sexual selection) might occur, there is expected to be significant evolutionary pressure to increase sperm velocity. In comparing sperm parameters of 18 species of muroid rodents, Tourmente *et al.* (2015a) found that those species exhibiting competitive breeding strategies produce faster swimming spermatozoa and higher sperm ATP content (Tourmente *et al.* 2015a). This was later demonstrated to be attributed to a greater reliance on OXPHOS versus glycolytic pathways and is likely an adaptation to differing levels of sperm competition (Tourmente *et al.* 2015b). Although horses are not strictly considered polyandrous, it is not uncommon for mares to be bred by non-dominant stallions, while avoiding detection by the harem's dominant stallion (McDonnell 2016). Mares have also been reported to breed with multiple stallions, possibly to confuse paternity, thus avoiding infanticide by a stallion believing the foal to be another stallion's offspring (Bartoš *et al.* 2018). These behaviours are expected to facilitate postcopulatory sperm competition and contributed to the evolution of fast swimming, OXPHOS-reliant spermatozoa that efficiently produce high levels of ATP enabling them to rapidly reach and fertilise the oocyte.

Lipid membrane peroxidation

The sperm plasma membrane is made up of both saturated fatty acids (FA) and long-chain PUFAs, which are the initial targets of ROS attack. Stallion spermatozoa have a high ratio of PUFA to saturated FA, and this ratio further increases during the breeding season (Aurich *et al.* 2018). Docosapentaenoic acid (DPA), which is the predominant fatty acid in equine spermatozoa (Parks & Lynch 1992), has also been shown to increase from the non-breeding to breeding season, causing a decrease in the overall omega 3:6 PUFA ratio (Aurich *et al.* 2018).

The high concentration of PUFA contributes to high levels of fluidity and elasticity, both of which are necessary for sperm–egg fusion events and consequently, fertilisation (Hosnedlova *et al.* 2017, Aurich *et al.* 2018). However, this renders stallion spermatozoa more susceptible to lipid membrane peroxidation, particularly when combined with the high concentrations of ROS produced as a by-product of OXPHOS (Aitken & Fisher 1994). Significant correlations between sperm ROS production and motility loss are largely explained by peroxidation of the lipid membrane, which leads to structural damage, changes in membrane fluidity and a loss of sperm function, including progressive motility and sperm–egg recognition (Aitken *et al.* 2010b, Gibb *et al.* 2014, Bromfield *et al.* 2015b, Kasperczyk *et al.* 2015, Hall *et al.* 2017). In addition, lipid peroxidation is associated with decreased sperm count, motility and percent morphologically normal cells *in vivo* and is one of the major contributors to oxidative and carbonyl stress (Shiva *et al.* 2011, Mohanty *et al.* 2016). Lipid peroxidation has been associated with numerous sperm deficiencies, and malondialdehyde (MDA), a marker of lipid peroxidation, has been reported as higher in the spermatozoa of oligozoospermic and azoospermic males, as well as asthenozoospermic and teratozoospermic males when compared to their normozoospermic counterparts (Shiva *et al.* 2011). Lipid peroxidation also affects processes of sperm–oocyte fusion through premature acrosome damage (Gomez *et al.* 1998) and sperm–egg recognition (Bromfield *et al.* 2015b), which render the spermatozoa incapable of fertilising the oocyte (Töpfer-Petersen *et al.* 2005).

Cytotoxic aldehydes are generated following the removal of hydrogen atoms from PUFA during lipid peroxidation. Examples of these include acrolein, 4-hydroxynonenol (4HNE) and to a lesser extent MDA. Once generated, these cytotoxic aldehydes can further disrupt the ETC by covalently binding to exposed proteins, thereby disrupting their functionality. Examples of this include the disruption of ATP production by the adduction of 4HNE to succinate-coenzyme Q reductase (Aitken *et al.* 2012a, Moazamian *et al.* 2015) and the adduction of 4HNE to the heat shock protein HSPA2; an essential component of the HSPA2/ARSA/SPAM1 protein complex in both human and stallion spermatozoa (Bromfield *et al.* 2015b). This complex is necessary for sperm–egg recognition, and, as a consequence 4HNE adduction renders the sperm cell incapable of achieving fertilisation (Bromfield *et al.* 2015a,b).

Acrolein, one of the most reactive of aldehydes (Moazamian *et al.* 2015), may be formed as a consequence of PUFA peroxidative processes or during the oxidation of polyamine metabolites by cellular enzymes (Alarcon 1970, Hales 1982), which are present in high concentrations within the male reproductive tract. As such, acrolein plays a role in human subfertility

(Moazamian *et al.* 2015), but whether this is the case in the stallion is yet to be ascertained.

Variation in fertility resulting from DNA damage

Recent research into idiopathic infertility has revealed strong links between DNA damage and decreased fertility, early embryo loss and birth defects (Aitken *et al.* 2010b, Chandra *et al.* 2012). There are numerous factors which lead to DNA damage, but one common underlying factor is incomplete protamination which leads to poor compaction of DNA during spermatogenesis, thus leaving DNA vulnerable to assault during times of heat stress and oxidative attack (Moorefield *et al.* 2004, Aoki *et al.* 2006, Zhang *et al.* 2006, De Iuliis *et al.* 2009, Mohanty *et al.* 2016, Ketchum *et al.* 2018).

DNA packaging

During early spermatogenesis, histones and protamines function by binding and packaging nuclear DNA. Histone-bound DNA sequences are loosely compacted and so the histone-to-protamine transition is an essential process in achieving adequate condensation of sperm DNA. This involves extensive chromatin reorganisation and compaction, whereby histones are firstly removed and replaced by transitional proteins, which are consequently replaced by protamines (Ward & Coffey 1991, Jha *et al.* 2017). In doing so, the DNA is more tightly compacted, allowing for streamlining of the sperm head, DNA stabilisation and crucially, protection of DNA from exogenous oxidative stressors (Aoki *et al.* 2006, Zhang *et al.* 2006, Ni *et al.* 2016). Any disruption to the composition of nuclear proteins will have profound effects on DNA integrity and therefore future fertilising potential and embryonic development (Szczygiel & Ward 2002, Zhang *et al.* 2006). The abnormal expression of protamines (P1 or P2) leads to aberrant protamine ratios, which are, in turn, associated with defects in spermatogenesis and spermiogenesis leading to conditions such as oligozoospermia, asthenozoospermia or teratozoospermia, particularly in relation to head morphology and diminished sperm penetration assay scores (Aoki *et al.* 2006). Indeed, Netherton *et al.* (2017) reported a 3-fold increase of P2 in poor quality spermatozoa isolated using density gradient centrifugation. In the stallion, three protamine variants have been identified; protamine 1 St1 and protamine 2 variants St2b and St2a (Pirhonen *et al.* 1989). Although the sequence of St2b and St2a are homologous, they differ in length and amino acid sequence which is thought to have result from gene duplication and successive point mutations (Pirhonen *et al.* 1989). However, since their discovery, no studies have yet indicated that stallion infertility is associated with aberrant protamine ratios.

Levels of the precursor protamine 'pre-P2' found in the maturing spermatozoon influences the P1/P2 ratio, with higher pre-P2 levels indicative of a failure in the histone-to-protamine replacement process (Torregrosa *et al.* 2006). These protamine aberrations may be corrected through the use of *in vitro* manipulations. In men, even within sperm populations which are deemed protamine deficient upon global assessments, there are populations of cells present with normal protamine content which can be selected by density gradient centrifugation for use in assisted reproductive procedures such as IVF and ICSI (Aoki *et al.* 2006). However within the equine industry, conventional equine IVF attempts have been largely unsuccessful (Leemans *et al.* 2016), ICSI is both costly and not readily available on a commercial basis, while such technologies are not permitted by the thoroughbred industry.

DNA under oxidative attack

The onset of oxidative stress has been attributed as the root cause of a significant proportion of the sperm DNA damage (De luliis *et al.* 2009). DNA damage, when measured using the sperm chromatin structure assay (SCSA), has been shown to correlate with morphological defects and indeed fertility in the stallion along with other livestock species (Lopez-Fernandez *et al.* 2008, Morrell *et al.* 2008, Kumaresan *et al.* 2017). The decreased DNA integrity that may be identified using SCSA can be caused by multiple, independent factors stemming from events that occur during spermatogenesis (Torregrosa *et al.* 2006). There are numerous ways by which ROS can cause DNA damage including alteration of nitrogenous base pairs, modification to DNA crosslinks, induction of DNA fragmentation as a result of strand breaks, and finally chromosomal rearrangements (Kemal Duru *et al.* 2000). ROS have been identified as one of the main factors in the induction of DNA damage and therefore subfertility in stallions (Baumber *et al.* 2003). The vulnerability of defective sperm cells to oxidative attack has been highlighted with strong correlations evident between defective protamination or chromatin remodelling and spontaneous 8-hydroxy-2'-deoxyguanosine (8OHdG) formation (De luliis *et al.* 2009).

Strong correlations have been reported between nuclear 8OHdG formation, a major product of oxidative DNA damage and a known marker of oxidative stress, and chromatin remodelling (measured using CMA3), DNA fragmentation (measured by the TUNEL assay), mitochondrial superoxide levels and the loss of mitochondrial membrane potential (De luliis *et al.* 2009). Sperm mitochondria play a crucial role in the oxidative stress cascade, with unscavenged mitochondrial ROS leading to the release of cytochrome C, resulting in caspase activation, apoptotic-like changes and cell death (Liu *et al.* 1996, De luliis *et al.* 2009). Furthermore, mitochondrial DNA is far more vulnerable to damage

than nuclear DNA, so much so that some have postulated that the mitochondrial genome may provide a very sensitive early biomarker of ROS imbalance (Sawyer *et al.* 2003, Aitken *et al.* 2010a). Owing to the increased dependence on mitochondrial ATP production by stallion spermatozoa (Gibb *et al.* 2014), it is anticipated that the stallion sperm mitochondrial genome may in the future provide a robust marker of fertility for diagnostic and management purposes.

Sperm proteins and their modifications as indicators of stallion fertility

A proteome is the subset of proteins expressed within an organism, a cell type or a disease process (Thompson *et al.* 2016). In addition to the sperm cell proteome, a plethora of proteins are acquired by spermatozoa from accessory sex gland secretions and from the epididymis during maturation and ejaculation (Amaral *et al.* 2014). As protein-to-protein interactions are responsible for the vast majority of cellular functions, proteomic analyses provide both an insight into the effectiveness of spermiogenesis and also an understanding of the interaction between sperm molecular functions and fertility (Baker *et al.* 2007, Aitken & Baker 2008, Oliva *et al.* 2009, Kwon *et al.* 2015a). The total protein content of the stallion ejaculate has been reported to range from 127 to 642 mg, while protein concentration ranges from 6.27 to 26.9 mg/mL (Novak *et al.* 2010). Swegen *et al.* (2015) identified over 1000 stallion sperm proteins, representing 975 genes. Of those, 372 proteins were involved in the cellular metabolic processes of fatty acid metabolism, glycolysis and OXPHOS, while 20 proteins were involved in defensive responses to oxidative damage. These include ROS scavengers superoxide dismutase and glutathione peroxidase (Swegen *et al.* 2015); although widely documented as the most important sperm antioxidants in other species, one report has suggested that their activity is limited in equine spermatozoa (Baumber & Ball 2005), with the most important antioxidants being the aldehyde dehydrogenase family of enzymes (Gibb *et al.* 2016).

Differential protein expression

In animal production systems, proteomic tools are being used to identify global protein biomarkers, and this approach has successfully facilitated the prediction of superior sires for use in selective breeding programmes in boars (Kwon *et al.* 2015b). In the equine industry, particularly the thoroughbred sector, the identification of proteomic biomarkers of fertility is under active investigation and is expected to facilitate the prediction of times of high and low fertility during the breeding season. This will allow the implementation of management strategies to maximise PCC rates and in turn, breeding efficiency. To date, several studies have

identified fertility biomarker candidate proteins from the spermatozoa of mice, men, livestock production animals, and to a lesser extent, stallions (Baker *et al.* 2005, Novak *et al.* 2010, Park *et al.* 2012, Kwon *et al.* 2015a,b, Swegen *et al.* 2015, Netherton *et al.* 2017). Indeed, at the time of publication of this review, a PubMed search using the terms 'stallion', 'sperm' and 'proteome' produced only six publications.

The expression of a number of proteins have been both positively (the glycolytic enzyme enolase (1) and negatively (voltage-dependent anion channel 2 and ubiquinol-cytochrome-c reductase complex core protein 2; UQCRC2) associated with bull fertility (Gitlits *et al.* 2000, Park *et al.* 2012). VDAC2 is associated with metabolite transportation, signal transduction and capacitation-related tyrosine phosphorylation (Arcelay *et al.* 2008, Liu *et al.* 2009) and is believed to be downregulated by the mitochondrial trifunctional protein, HADHA, which has also been positively correlated with boar fertility (Kwon *et al.* 2015a). UQCRC2, a mitochondrial protein, has been associated with both oxidative stress and ROS production, with UQCRC2 deficiency shown to increase ROS generation (Aguilera-Aguirre *et al.* 2009). Owing to the susceptibility of stallion spermatozoa to oxidative attack, and their reliance on OXPHOS as opposed to glycolysis, it will be of interest to investigate the expression of both enolase 1 and UQCRC2 during times of high and low fertility.

Spermadhesins, a family of secretory proteins, are expressed primarily in the seminal plasma and following ejaculation become bound to the sperm surface. It is postulated that the heparin and carbohydrate-binding ability of these proteins facilitates sperm–oocyte fusion (Topfer-Petersen *et al.* 1998, Töpfer-Petersen *et al.* 2005) and as such they have been the focus of numerous studies. Spermadhesins are abundant in the ejaculates of boars, whereas the diversity and expression of these proteins are greatly reduced in other species such as cattle, horses and humans (Töpfer-Petersen *et al.* 2005). Despite this, the similarity in the amino acid sequences of boar, bull and stallion spermadhesins can range from 40% up to 98% (Topfer-Petersen *et al.* 1998). The fertility status of production animals has been linked to the expression of such proteins in numerous studies, with spermadhesin Z13 (SPADH2) differentially expressed in the spermatozoa of fertile, subfertile and infertile bulls (Somashekar *et al.* 2017). While the exact mechanism by which SPADH2 affects fertility is yet to be elucidated, spermadhesins AQN-3 and AWN have been associated with litter size in boars (Kwon *et al.* 2015b, Somashekar *et al.* 2017), and as the amino acid sequence of stallion spermadhesin protein HSP-7 shares 98% similarity with boar AWN (Reinert *et al.* 1996, 1997), it is conceivable that they share a common underlying function (Sanz *et al.* 1992, Reinert *et al.* 1997). Equine HSP-7 is a carbohydrate-binding protein and functions in the zona-binding interaction (Reinert *et al.* 1996); therefore,

the expression of this protein may be another possible marker of fertility in stallions, warranting further investigation.

Positive associations between seminal plasma IGF1 concentrations and stallion fertility have also been reported (Macpherson *et al.* 2002, Novak *et al.* 2010). Novak *et al.* (2010) proposed the measurement of total ejaculate IGF1 content could be the most simple and effective measure of stallion fertility. This is a useful predictor in the area of artificial insemination, but its use in the natural breeding scenario is confounded by the fact that this calculation requires knowledge of both the IGF1 concentration (per mL), as well as the total volume of the ejaculate; this is not known even if a post-breeding semen 'dismount sample' is collected as is often the case in the thoroughbred.

Seminal plasma proteins such as kallikrein-1E2, clusterin and specificity proteins SP1 and SP2 have been reported as being negatively associated with fertility, while both clusterin and SP1 are positively associated with ejaculate volume in stallions (Novak *et al.* 2010). SP1 and SP2 are part of the specificity protein (SP) family, functioning primarily in transcriptional regulation (Bouwman & Philipsen 2002, Kolell & Crawford 2002). SP shares a DNA-binding domain and are responsible for the expression of a wide variety of genes, particularly SP1 (Bouwman & Philipsen 2002, Kolell & Crawford 2002). Given that up to 25% of negative pregnancy diagnoses in horses may be attributable to early embryo death (Ball *et al.* 1989), this family of proteins are of particular interest, as murine studies have demonstrated that SP1 and SP2 are essential for normal embryonic development (Bouwman & Philipsen 2002, Baur *et al.* 2010).

Clusterin is a chaperone-like protein involved in protein-to-protein reactions, lipid exchange and membrane remodelling during maturation (Law & Griswold 1994, Griffiths *et al.* 2009). Interestingly, in bulls an inverse relationship was reported between sperm clusterin content and non-return rates, conception rates and sperm motility, while a positive relationship with percentage morphological abnormalities (Ibrahim *et al.* 2000). Similarly, expression of the clusterin gene was significantly increased in the semen samples of infertile men, compared to fertile cohorts, as reported by Zalata *et al.* (2012). In stallions, clusterin is the second most abundant protein secreted by the epididymis, indicating the importance of its role during epididymal maturation (Fouchecourt *et al.* 2000). However, there is a paucity of literature focused on elucidating the exact role of clusterin within the stallion ejaculates; therefore, its effect on sperm functionality remains unknown.

The sperm proteins malate dehydrogenase (MDH), fumarate hydratase, citrate synthase, α -enolase and dihydrolipoamide dehydrogenase are positively associated with first cycle conception rate in stallions (Novak *et al.* 2010). As first cycle conception rate is deemed to be a good indicator of relative fertility, it is

believed that such proteins could be used as biomarkers or predictors of fertility. MDH, fumarate hydratase and citrate synthase are enzymes involved in carbohydrate metabolism. Although stallion spermatozoa predominantly rely on OXPHOS for motility, carbohydrate metabolism may have uncharacterised roles in sperm function or survival. Indeed, improved carbohydrate metabolism may facilitate the increased use of glycolysis. Incubation of stallion spermatozoa with rosiglitazone, an antidiabetic compound, seems to increase their use of glycolysis, resulting in a decreased reliance on OXPHOS pathways, and thereby reducing ROS production and increasing longevity (Swegen *et al.* 2016).

Calmodulin (CALM1) has been identified as a possible biomarker of high fertility in boars (Kwon *et al.* 2015b) and bulls (Somashekar *et al.* 2017). CALM1 is an intermediate calcium-binding messenger protein, which interacts with ten key proteins including those involved in the regulation of calcium (Ca^{2+}), mediation of the centrosome, protein kinases and cytokinases (Somashekar *et al.* 2017). Ca^{2+} is one of the most important ions in the maintenance of sperm cell function. Ca^{2+} is particularly important in motility as a rise in Ca^{2+} stimulates the Ca^{2+} sensory proteins calaxins, which in turn inhibit dynein activity in the cell axoneme causing high amplitude asymmetric flagellar hyperactivation (Ho *et al.* 2002, Shiba *et al.* 2008, Mizuno *et al.* 2009, 2012). Furthermore, Ca^{2+} ions trigger the acrosome reaction, thereby enabling oocyte penetration and fertilisation (Yanagimachi 1970, Hong *et al.* 1984, Publicover *et al.* 2007, Kasperczyk *et al.* 2015). In stallion spermatozoa CALM1 is localised to the acrosome and flagellar principal piece and is involved in the regulation of sperm motility, the onset of tyrosine phosphorylation and thus capacitation (Gonzalez-Fernandez *et al.* 2012, Lasko *et al.* 2012), while in bulls, the under-expression of CALM1 was associated with the spermatozoa of subfertile and infertile bulls (Somashekar *et al.* 2017). Therefore, it would be expected that any change in the expression of this protein would result in fluctuations in fertility, but this hypothesis is yet to be confirmed.

Angiotensin-converting enzyme (ACE) has been identified in the testes and seminal plasma, with particularly high concentrations identified in the plasma membrane of ejaculated stallion spermatozoa (Ball *et al.* 2003). ACE is a dipeptidylcarboxypeptidase and while the specific role of ACE in sperm function is yet to be elucidated, it is thought that ACE functions in capacitation and acrosome reaction (Sabeur *et al.* 2000, Ball *et al.* 2003). Testis Ace knockout mice experience a high rate of fertilisation failure, with a significant reduction in both oviductal sperm transit and zona binding (Hagaman *et al.* 1998). In stallions, the haplotype of the ACE gene significantly influences both the paternal and embryonic contribution to pregnancy, measured by PCC rates (Giesecke *et al.* 2011). ACE may prove as a valuable

marker of fertility; however, further research is required to determine the exact mechanism by which ACE affects sperm function and to investigate its expression, along with the expression of other proteins discussed earlier, during times of high and low fertility. Figure 1 illustrates the journey of the spermatozoon from cell generation to fertilisation, highlighting the molecular pathways and signalling proteins associated with sperm maturation, ejaculation and fertilisation.

While it is unlikely that a single protein can serve as a biomarker for subfertility of all aetiologies, further work is expected to reveal a series of reliable correlations between specific proteins and conception rates; these can then be developed into a robust multi-biomarker diagnostic platform for the early detection of stallion subfertility.

Post-translational modifications

The high level of molecular diversity exhibited by the proteome is determined by numerous factors including the post-translational modification (PTM) of proteins (Samanta *et al.* 2016). As the spermatozoon has a reduced ability to synthesise proteins, the primary method in which functionality is acquired is via PTMs (Blaquier *et al.* 1988, Ross *et al.* 1990). The latter chemically alters protein properties by covalent processing events, such as the addition of modified groups to amino acid side chains or by proteolytic cleavage, and are generally catalysed by substrate-specific enzymes (Mann & Jensen 2003, Samanta *et al.* 2016). These enzymes regulate the distribution, stability and function of proteins (Samanta *et al.* 2016). Epididymal maturation processes are essential for the creation of functional sperm cells (Baker *et al.* 2005). In the stallion, Fouchecourt *et al.* (2000) reported that at least 117 proteins are secreted into the epididymal lumen, many of which are capable of inducing the PTM of sperm proteins. Protein activity state, turnover, localisation and protein-to-protein interactions are governed by PTMs; therefore, the importance of such processes cannot be underestimated (Mann & Jensen 2003). Table 1 illustrates the functional significance of different types of PTMs for normal cell metabolism: for example, phosphorylation is important for cell signalling while methylation is required for the regulation of gene expression.

Oxidative stress leads to the incorrect folding or cleavage of proteins, conformational changes to protein structure and folding, protein carbonylation and failure of vital processes such as phosphorylation and glycosylation. Under normal conditions, phosphorylation involves the alteration of protein structures via the introduction of covalently bound phosphate groups to the polypeptide chain (Samanta *et al.* 2016). It is one of the major PTMs responsible for important sperm functions, from maturation through to the acrosomal reaction and fertilisation

Figure 1 Journey of the equine spermatozoon from cell generation through to fertilisation; the effects of disruptions to cell processes, and the proteins implicated in each stage of development, involving function, signalling and stages when most vulnerable to damage.

(Sakkas *et al.* 2003). A series of *in vitro* and *in vivo* experiments carried out by Gonzalez-Fernandez *et al.* (2012) have revealed that the onset of tyrosine phosphorylation in stallion spermatozoa is governed primarily by pH. The lower pH, together with the Ca^{2+} content of stallion semen, prevents the onset of phosphorylation. However, once within the uterine lumen, which is at a higher pH, a series of biochemical events unfold resulting in the onset of tyrosine phosphorylation and capacitation. The same conclusion has also been reached for human spermatozoa (Aitken *et al.* 1998).

The process of acetylation is critical for correct DNA packaging and chromatin condensation. Ketchum *et al.* (2018) have recently published that the histone H4 lysine acetylation event, H4K16ac, is the key marker in initiating the transition of nucleoproteins from histone-to-protamine structures in stallion spermatozoa. Conversely, protein carbonylation is not presently known to have beneficial roles in sperm functionality. This PTM is mediated by ROS, involving the oxidation of proteins, and is therefore negatively associated with sperm quality (Suzuki *et al.* 2010, Shiva *et al.* 2011). Oxidative stress can cause total or partial inactivation

Table 1 Functions of select post-translational modifications (adapted from (Mann & Jensen (2003)).

PTM type	Functions
Phosphorylation	Activation and inactivation of enzyme activity, modulation of molecular interaction, signalling
Acetylation	Protein stability, protection of N terminus, regulation of protein–DNA interactions
Acylation (fatty acid modification)	Cellular localisation and targeting signals, membrane tethering, mediator of protein–protein reactions
Methylation	Regulation of gene expression
Glycosylation	Excreted proteins, cell–cell recognition/signalling
GPI Anchor	Membrane tethering of enzymes and receptors
Hydroxyproline	Protein stabilisation and protein–ligand interactions
Disulphide Bond Formation	Intra- and intermolecular crosslink, protein stability
Ubiquitination	Destruction signal

PTM, post-translational modifications.

of proteins as a consequence of conformational changes or damage to the polypeptide chain (Mohanty *et al.* 2016). Monitoring PTMs may provide useful information pertinent to fertility; however, no studies have focused on the mechanisms by which aberrant PTMs affect fertility in stallions and whether or not they can be used as biomarkers of fertility.

Lipid peroxidation together with protein carbonylation has been indicated as a biomarker for fertility, with the model reported by Mohanty *et al.* (2016) predicting the fertility status of fertile men versus recurrent pregnancy loss sufferers with 97% accuracy. As previously discussed, stallion spermatozoa produce high levels of ROS owing to their use of the OXPHOS pathway, and therefore, are predisposed to oxidative stress affecting lipids, proteins and DNA. The root cause and indeed full effects of aberrant PTM on sperm functionality and overall fertility have not yet been examined. Investigation into these mechanisms and the failure of normal protein function will provide pathways to elucidating how management regimens can affect stallion fertility, leading to enhanced production systems. Table 2 illustrates the most important diagnostic tools employed for investigating and predicting stallion fertility, along with the advantages and disadvantages of each assessment type.

Role of RNA species in stallion sperm function

The spermatozoon was previously described as being transcriptionally and translationally silent; however, new research has provided compelling evidence for

the presence of sperm RNAs within the cell (Das *et al.* 2013, Ing *et al.* 2014). Ostermeier *et al.* (2002) conducted one of the first studies to reveal the presence of several thousand messenger RNAs (mRNA) in human spermatozoa. Since then there has been extensive research focused on the sperm transcriptome in livestock animals and to a lesser extent in stallions (Das *et al.* 2013, Jevsinek Skok *et al.* 2013, Ing *et al.* 2014). Originally it was believed that sperm RNAs were simply residues of spermatogenesis and served no function in cellular processes (Das *et al.* 2013). Studies have since demonstrated that mRNAs such as clusterin, PRM1 and PRM2 mRNA are delivered to the oocyte by spermatozoa upon fertilisation, thus contributing to zygotic and early embryonic development (Kempisty *et al.* 2008). Yuan *et al.* (2016) demonstrated that embryos generated from the sperm of *Dicer* conditional knockout mice had defective development to blastocyst stage; however, such defects were rescued following injection with purified sperm RNAs. In stallions, the use of sperm transcriptome analysis is now being investigated as a method of fertility biomarker discovery, and as a diagnostic tool for fertility assessments (Das *et al.* 2013).

A number of RNA species, including mRNA, lncRNA, rRNA and miRNA, have been characterised in stallion spermatozoa (Das *et al.* 2013, Ing *et al.* 2018). Approximately 11,500 gene transcripts have been detected in the stallion testes (Coleman *et al.* 2010, Das *et al.* 2013); 6500 of these are also present in stallion sperm, a ratio similar to that previously reported in men (Ostermeier *et al.* 2002). Of these, 60 transcripts are upregulated in spermatozoa compared to testes,

Table 2 Diagnostic tools for the investigation and prediction of stallion fertility.

Assessment	Advantages	Disadvantages
DNA damage: sperm chromatin dispersion	<ul style="list-style-type: none"> Simple and inexpensive High repeatability and accuracy Strong correlation with SCSA and TUNEL assays 	<ul style="list-style-type: none"> Initial investigations reported no correlation with pregnancy outcome Use in stallion fertility assessments yet to be investigated
DNA damage: sperm chromatin structure assay (SCSA)	<ul style="list-style-type: none"> Low intra- and inter-laboratory variation; robust DFI assay Predictor of pregnancy success and fertility in other species Correlations reported between stallion fertility and SCSA 	<ul style="list-style-type: none"> Flow cytometry required Proficiency of SCSA uncovering full extent of DNA damage questioned Reliability as a predictor of stallion fertility fluctuations yet to be investigated
Oxidative DNA Damage: 8OHdG	<ul style="list-style-type: none"> 8OHdG identified as an early biomarker of oxidative damage 8-OHdG negatively correlated with fertility, and sperm quality in humans 	<ul style="list-style-type: none"> Flow cytometry required Several methods and commercial kits available; variable results Few reports implicating a relationship with stallion fertility
Morphological assessment	<ul style="list-style-type: none"> Simple and inexpensive Correlations reported between percent morphologically normal spermatozoa and stallion PCC rates Inferred as an indicator of DFI 	<ul style="list-style-type: none"> Analysis is subjective Few studies investigating predictive value of morphological assessment with stallion fertility fluctuations
Proteomic assessments	<ul style="list-style-type: none"> Provides insights into protein-to-protein interactions and effectiveness of spermiogenesis Highly reliable in identification of biomarkers of fertility Used in the prediction of superior sires in boar breeding programmes 	<ul style="list-style-type: none"> Costly and equipment not always readily available No research carried out thus far into the differential expression of proteins during times of high and subfertility in stallions

while 165 transcripts have been detected in the sperm cell only, and pertain to genes involved in processes of ion, nucleotide and chromatin binding (Das *et al.* 2013). Therefore, stallion sperm mRNAs are not simply a remnant of the spermatozoon's past life as a diploid cell, but play active roles in sperm–egg interactions, fertilisation and early embryonic development (Das *et al.* 2013). Initial research into the use of mRNA as biomarkers of fertility has revealed four mRNAs (CATSPER1, OAZ3, aromatase (CYP13A1) and oestrogen receptor ESR1) which are lower in concentration in high quality spermatozoa when compared to low-quality sperm populations (Ing *et al.* 2014).

Spermatozoa were originally thought to be rRNA depleted (Ostermeier *et al.* 2002); however, studies have since proven the contrary in stallion spermatozoa (Das *et al.* 2013). During spermiogenesis, full-length rRNAs are degraded via selective cleavage in order to ensure translational arrest and thus prevent the synthesis of false proteins (Das *et al.* 2013).

Preliminary findings from Ing *et al.* (2018) have uncovered over 11,000 unique lncRNAs; 166 of which are differentially expressed between low and high-quality stallion spermatozoa. Ing *et al.* (2018) also identified 159 mature miRNA sequences; eight of which are elevated in high-quality spermatozoa. Although only preliminary findings, such numbers of RNA species are almost double those previously identified by Das *et al.* (2013). MiRNA are short non-coding RNAs and function in the post-transcriptional regulation of mRNA by destabilising or suppressing translation, thus impeding protein production (Jevsinek Skok *et al.* 2013, Schrimpf *et al.* 2014). Research into sperm miRNA expression in mice revealed that the miRNA cluster, MiR-880, plays a significant role in early embryonic development and gene regulation, as embryos generated using testicular and cauda sperm populations, which carry abundant MiR-880, developed normally, while embryos generated using sperm isolated from the caput epididymis, which are deficient in miR-880, failed soon after implantation (Conine *et al.* 2018). Das *et al.* (2013) previously identified three stallion sperm miRNAs (MIR34B, MIR34C and MIR449A) as being critical for gene expression and early embryonic development. MiRNAs are also potentially involved in the regulation of steroid synthesis during spermatogenesis, as these molecules have been indicated to function in the regulation of both Leydig and Sertoli cells in stallions (Schrimpf *et al.* 2014). Recently, Tsatsanis *et al.* (2015) identified miR-155 as marker of subfertility in men; therefore, it is conceivable that sperm miRNA expression may be developed in the future as a diagnostic tool reflective of sperm fertilisation capacity. However, such research, especially with regard to the stallion is in its early stages, and further research is required to elucidate the exact functions and roles of such non-coding RNAs in stallion subfertility.

Single nucleotide polymorphisms (SNPs) can affect the maturation, function, structure or expression levels of miRNAs (Mishra *et al.* 2008). Any disruption to miRNA function will have further downstream effects at the gene expression level. Schrimpf *et al.* (2014) identified three SNPs that significantly affect the estimated paternal contribution to pregnancy rates in Warmblood stallions. Authors proposed that the expression level of phospholipase C zeta 1 (PLCz1), a stallion fertility locus, may be affected when the miRNA motif is removed by such SNPs. Meanwhile Jevsinek Skok *et al.* (2013) reported that miRNA polymorphisms have a profound effect on a wide range of phenotypic traits in livestock animals, including that of the horse. However, at the time of this study, only one miRNA polymorphism was identified within the horse genome, in comparison to 125 in cattle and 92 in chicken, which authors attribute to a lag in equine genomic focused research, rather than a lack of genetic variability (Jevsinek Skok *et al.* 2013).

The role of sperm RNAs, particularly miRNA and lncRNA are still somewhat undetermined. However, new research is continuously emerging, demonstrating that such RNAs are essential for sperm function, fertilisation and early embryo development. Furthermore, research has also focused on the differential expression of RNAs, recently Jiang *et al.* (2016) reported over 7700 lncRNAs and over 6000 mRNAs which were differentially expressed between the sperm of diabetic mice and controls. Authors revealed through GO and pathway analysis that the functions of such mRNAs were closely associated with many spermatogenic processes such as OXPHOS, glucose metabolism and lipid metabolism (Jiang *et al.* 2016). These findings are extremely relevant and may prove useful in the stallion to determine the link between fertilising potential, fertility fluctuations and RNA expression.

Conclusion

Deciphering the metabolic strategies employed by stallion spermatozoa has heralded the ability to use ROS production, antioxidant capacity and DNA damage measurements as markers of sperm quality, predictors of fertility fluctuations and indeed, diagnostic techniques for the investigation of stallion subfertility. There has been increased focus on the use of proteomic tools in fertility biomarker discovery, with several sperm biomarker proteins identified in livestock species; yet, stallion sperm proteomic studies thus far have been largely descriptive in nature. Large-scale, quantitative analyses targeting the full proteome are needed in order to develop truly useful biomarker panels which are relevant across the breeding industry.

Even less well studied are the roles of RNA species in stallion sperm function. Future research must focus on elucidating the link between sperm RNA species and fertility, and in particular, whether non-coding

RNAs participate in the spermatozoon's acquisition of fertilising capacity or contribute significantly to embryo development. Such studies will potentially provide additional means for predicting the breeding prospects of stallions, which will be crucially important for planning the breeding 'careers', along with optimal welfare outcomes of these animals. The equine breeding industry, with its accurate recording of PCC rates and closely managed breeding systems, provides an excellent opportunity for meaningful and thorough investigation of the roles of emerging molecular mechanisms in sperm function and fertility.

Declaration of interest

Laureate Professor John Aitken is a member of the editorial board of *Reproduction*. The other authors have nothing to disclose.

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Author contribution statement

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