Proteomics and metabolomics in cow fertility: a systematic review

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Abstract

Cow subfertility is a multi-factorial problem in many countries which is only starting to be unravelled. Molecular biology can provide a substantial source of insight into its causes and potential solutions, particularly through large scale, untargeted omics approaches. In this systematic review, we set out to compile, assess and integrate the latest proteomic and metabolomic research on cow reproduction, specifically that on the female reproductive tract and early embryo. We herein report a general improvement in technical standards throughout the temporal span examined; however, significant methodological limitations are also identified. We propose easily actionable avenues for ameliorating these shortcomings and enhancing the reach of this field. Text mining and pathway analysis corroborate the relevance of proteins and metabolites related to the triad oxidative stress-inflammation-disease on reproductive function. We envisage a breakthrough in cattle reproductive molecular research within the next few years as *in vivo* sample techniques are improved, omics analysis equipment becomes more affordable and widespread, and software tools for single- and multi-omics data processing are further developed. Additional investigation of the impact of local oxidative stress and inflammation on fertility, both at the local and systemic levels, is key towards realising the full potential of this field.

Reproduction (2020) 160 639–659

Introduction

Dairy cow subfertility

Dairy cow reproductive performance has been declining in many countries around the world, whose aetiology seems to be related to changes in cow genetic selection, physiology and management associated with increased milk production (LeBlanc 2010).

Substantial economic losses, management difficulties and animal welfare concerns (Lucy 2001) have motivated a great deal of research to unravel the mechanisms of reduced fertility. Considerable progress to halt the decline in dairy cow fertility has been made in recent years using genetics approaches (Berglund 2008).

To understand the factors underlying this and other physiological conundrums, the trend in clinical and veterinary biochemistry has shifted in the last 30 years from investigating a few molecules in classical studies towards high-throughput methods (commonly referred to as 'omics') analysing hundreds or thousands of compounds.

Improvements in both equipment and data processing have increased the popularity and volume of proteomics and metabolomic studies. They are closer to phenotype than genomic traits and tend to better inform the organism's functional state, while also

reflecting genetic factors (Nagana Gowda & Raftery 2017). Moreover, post-translational regulation processes hinder quantitative predictions of protein levels based on transcript abundance, and therefore proteomics offers unique advantages over transcriptomics (Arnold & Frohlich 2011).

The relationship between the composition of blood and reproductive biofluids is complex, with certain blood proteins transferred to the uterine fluid (UF) through the uterine epithelium, but secretions from the endometrium being crucial for pregnancy success (Gray *et al.* 2002). Faulkner *et al.* (2012) showed that many proteins are differentially abundant in bovine UF compared to plasma. The relevance of integrating molecular research from different reproductive organs is further illustrated by Hunter *et al.* (2011) description of the mechanisms by which follicular fluid (FF) contributes to the composition of oviduct fluid (OF), as well as the exchange of fluids between OF and UF at the utero-tubal junction. Peritoneal fluid also seems to facilitate interactions among different parts of the reproductive tract (Hunter *et al.* 2007).

Systemic determinants of bovine fertility have been extensively discussed by Garnsworthy *et al.* (2008) with a focus on the interaction between feeding, lactation status, metabolic balance and hormones, and are not discussed in this review.

Aims of this study

Reproductive success depends on three main elements, namely paternal (comprehensively reviewed by Ceciliani *et al.* 2017), maternal, and the embryo itself (Artus *et al.* 2020).

Although the maternal environment is the focus of this work, molecular research into the developing embryo is also featured. The relevance of this distinction between maternal and embryo factors is illustrated by studies trying to assess their relative effects. A previous meta-analysis of 46 sets of data comprising 4560 recipient cows using embryo transfer only, as well as animals inseminated and concurrently receiving embryo transfer, demonstrated clear independent effects of embryos and recipient cows on embryo survival to parturition (McMillan *et al.* 1998).

Insight from transcriptomics studies, in this area, has been reviewed by Forde and Lonergan (2012) and Ulbrich *et al.* (2013). Thus, this work is aimed at proteomics and metabolomics with only occasional comparisons to transcriptomics data.

To the best of our knowledge, a comprehensive systematic review that consolidates research in this field is lacking in cows. This systematic review thus has two main aims: to identify the main trends and gaps in research of the bovine female reproductive tract, and to suggest strategies to boost the impact and reach of this field.

Results and discussion

Search and study inclusion

This study was designed following PRISMA guidelines for systematic reviews and meta-analyses (Moher *et al.* 2009). The selection criteria and steps undertaken are depicted in Fig. 1 and described in detail in Supplementary Section 1 (see section on supplementary materials given at the end of this article). The initial search resulted in 1730 potentially suitable articles. Analysis of the titles led to the removal of 1567 papers, including repeated hits, studies targeting male reproduction and articles on topics out of scope. A second stage of selection was based on the information in the abstract, which resulted in a further 54 studies being excluded for one of three reasons: they either presented no original results (i.e. reviews), did not investigate reproductive organs or tissues, or contained overlapping information with an included paper (data reports or abstracts from proceedings). Out of the 109 articles left, 20 studies were excluded that were either purely genomics or transcriptomics, or reported less than six proteins or metabolites. Based on the pilot trial, articles investigating exclusively embryo culture media or late pregnancy and birth complications were only marginally related to the rest of the selected studies. Discrepancies between *in vivo* and *in vitro* results using

Figure 1 Search strategy and parameters according to the PRISMA guidelines.

cell cultures were also noted and therefore an additional 17 studies were excluded for a final number of 72.

Table 1 is adapted from the master table upon which descriptive statistics were based. It presents an overview of the whole set, intended for the reader to locate relevant information at a glance. Supplementary Table 1 contains more detailed descriptions of each study included, classified according to the tissue or organ target: (1) endometrium, (2) embryo, (3) FF and oocytes, and (4) uterine and oviduct fluid (UF and OF, respectively).

Thirty-seven studies (52%) reported proteomic results, whereas 34 articles presented metabolomics data (47%), and 1 reported both (1%). Of the papers reviewed, 15 described transcriptomics results in addition to either proteomics or metabolomics, which provided more comprehensive and sounder biological insight in the phenomena investigated. A wide range was observed in the number of compounds measured (quartiles: 18, 53, 653).

Technical considerations

Studies in cattle tend to have a smaller sample size than humans or mice, due to both logistic and economic reasons (cost of maintenance and space required for large animals). The average sample size in this analysis was reasonably large at 28, although more replicates would increase statistical power and reliability of the trends reported.

As an overarching trend of this review, at first sight, many studies lend themselves to direct comparisons or integration. However, many confounding factors complicate this prospect. These include genetic makeup (milk production and health traits), diverse measurements of reproductive performance, climate, management conditions, including using animals in working farms (which may restrain or affect the design and execution of the experiments (Oenema *et al.* 2001)), as well as variation in sampling methods.

Breed and genetic makeup

Perhaps because of the assumed genetic homogeneity among the main cow breeds, this factor often goes unmentioned in articles. However, physiological differences between predominantly beef (Simmental, Angus, Charolais, Limousine) and dairy (Holstein or Holstein-Friesian) cattle (Hayes *et al.* 2009) or between those and zebu-type breeds (Carvalho *et al.* 2008) make it imperative to provide this information when presenting results, omics or otherwise. Even cows classed under the same breed name (Holstein) kept together under identical husbandry conditions were shown to differ significantly in milk production, health and reproductive traits (Horan *et al.* 2004). Gegenfurtner *et al.* (2019a,b) provided evidence of clear differences in the proteomic

composition of UF and OF between cows of different breeds (Holstein vs Montbéliarde) and between Holstein cows with divergent genetic merit for fertility.

Twenty-five articles investigated temperate climate beef cows of European origin, either pure or crossbred. Dairy cows were studied in 19 papers. Zebu/Nellore/Brahman (*Bos taurus indicus*) were researched in six studies. The remaining 23 studies did not provide any detail on the breed or type of cow used. Most of these latter studies sourced the animals from abattoirs.

Physiological conditions and management

Except for the few studies using either cow with endometritis or cystic follicles, all other articles investigated animals without clinical signs of disease. Further to those, a significant part of the studies relied on samples obtained from abattoirs, either for oocytes to generate embryos or as a source of samples, which usually means that little or no information is known about the animals (age, health, management conditions).

Management factors such as housing and feeding regime, time of the day and physiological well-being or stress are confounding factors that may well prevent detecting true differences in the parameters of interest (Nagana Gowda & Raftery 2017).

Related to the above, agreeing on a standard animal to allow comparisons when investigating the effects of diseases is imperative, especially considering that environmental and husbandry conditions vary widely at country, farm and herd levels (Philipsson *et al.* 2011).

Currently, there is no simple definition of a healthy animal and the normal concentration range of metabolites or proteins. Subclinical conditions may be ubiquitous and effectively impossible to diagnose using the current standard techniques (Ametaj 2017) and the distinction between clinical and subclinical conditions depends heavily on the choice of testing method (Helfrich *et al.* 2020). Therefore, we propose that results reported for either clinical or subclinical states deserve consideration, especially under the premise that both processes substantially affect fertility (McDougall 2001, McDougall *et al.* 2011).

In summary, establishing a healthy standard for comparisons is daunting, but its usefulness, as demonstrated in clinical chemistry (Goldansaz *et al.* 2017) showcases its relevance. This is further discussed in Section 2.2.4.

Sampling or chemical processing

The review by Velazquez *et al.* (2010) comments on advantages and disadvantages of the most typical techniques for sampling of OF and UF (flushing, aspirating and cannulation). The same work recommends *in vivo* sampling instead of postmortem and stresses the necessity to inhibit proteases or quench metabolism by snap freezing to minimise changes post-sampling (Velazquez *et al.* 2010).

Table 1 Studies included, sorted by tissue investigated.

References	Collection day	Sample	Omics	Keywords
Embryo				
Banliat <i>et al.</i> 2019b	7	Embryo (<i>in vitro</i>)	M	Effect of hormone treatments
Demant <i>et al.</i> 2015	5, 7	Embryo (<i>in vitro</i>)	P	Proteome changes in early development
Deutsch <i>et al.</i> 2014	1, 1.5, 2	1, 2 and 4-cell (<i>in vitro</i>)	P	Development; oocytes as reference
Goncalves <i>et al.</i> 2016	0, 2, 3, 7	Oocytes, embryos (<i>in vitro</i>)	M	MS-imaging to distinguish embryo stages
Hemmings <i>et al.</i> 2012	7, 8, 9	Oocytes, embryos (<i>in vitro</i>)	M	Modelling oocyte competence
Jensen <i>et al.</i> 2014a	8	Embryo (<i>in vitro</i>)	P	Descriptive, two embryonic tissues
Jensen <i>et al.</i> 2014b	13	Embryo (<i>in vitro</i>)	P	Different stages of embryo development
Massicotte <i>et al.</i> 2006	0–2.5	2, 4, 8-cell (<i>in vitro</i>)	P	Maternal housekeeping proteins
Sturmei <i>et al.</i> 2010	7	Embryo (<i>in vitro</i>)	M	Embryo sex-metabolism <i>in vivo</i> vs <i>in vitro</i>
Endometrium				
Belaz <i>et al.</i> 2016	Oestrus: 4, 7	Endometrium, oviduct	M	Lipidome profiling of uterus and oviduct
Berendt <i>et al.</i> 2005	Preg + Oestrus: 18	Endometrium	P	Monozygotic twins: pregnant vs oestrus
Choe <i>et al.</i> 2010	Oestrus: 0–4	Endometrium	P	Endometritis
Giergiel <i>et al.</i> 2018	Oestrus	Endometrium	P	Heifers vs calves
Groebner <i>et al.</i> 2011a	Preg: 12, 15, 18	Endometrium	M, (T, P)	Kynurenine metabolic pathway
Zhang <i>et al.</i> 2017	21–35 dpp	Endometrium (plasma)	P	Endometritis
Follicular fluid and oocytes				
Aller <i>et al.</i> 2013	Oestrus: 4, 13	FF (plasma)	M, (P)	Small vs large follicles and FF vs plasma
Annes <i>et al.</i> 2019	NA	FF and oocytes	M, (T)	Lipids of oocytes and FF in follicles
Bender <i>et al.</i> 2010	Across oestrus	FF (plasma)	M	FF vs plasma, subordinate vs main follicle
Bhojwani <i>et al.</i> 2006	0, 0.5, 1	Oocytes (<i>in vitro</i>)	P	Phosphorylation during <i>in vitro</i> maturation
Forde <i>et al.</i> 2016a	Oestrus	FF (plasma)	M	Effect of lactation on FF composition
Guerreiro <i>et al.</i> 2018	Dioestrus	FF	M	Higher, normal, and lower oocyte producers
Leroy <i>et al.</i> 2004a	NA	FF (plasma)	M	Metabolites in FF re follicle size and blood
Leroy <i>et al.</i> 2004b	16, 46 dpp	FF (plasma)	M	Metabolites in FF re time after calving
Leroy <i>et al.</i> 2005	16, 46 dpp	FF and oocytes	M	Effect of fatty acids on <i>In vitro</i> oocyte maturation
Maniwa <i>et al.</i> 2005	Oestrus	FF	P	Cystic vs healthy follicles
Matoba <i>et al.</i> 2014	NA	FF, follicle cells	M, (T)	FF good and bad embryos
Memili <i>et al.</i> 2007	NA	Oocytes, cumulus cells	P	Descriptive: germinal vesicle oocytes
Moore <i>et al.</i> 2015	Oestrus: 7	FF (plasma)	M	Genetic merit for fertility
Orsi <i>et al.</i> 2005	Across oestrus	FF, ECM	M	FF vs embryo culture medium
Renaville <i>et al.</i> 2010	Dioestrus	FF (plasma)	M	Active ((E2)/(P4) > 1) vs inactive follicles
Rodrigues <i>et al.</i> 2019	NA	FF	P, (T)	EVs in FF protect embryos from heat stress
Zachut <i>et al.</i> 2016	Pre-ovulatory	FF	P	Repeat breeders vs control (normal)
Uterine and oviduct fluid				
Alavi-Shoushtari <i>et al.</i> 2006	Across oestrus	UF (plasma)	P	Globulins and albumin
Almiñana <i>et al.</i> 2017	Oestrus: 1–5	OF (EVs)	P	EVs in OF and <i>in vitro</i> oviduct culture
Almiñana <i>et al.</i> 2018	Across oestrus	OF (EVs)	P, (T)	EVs in OF; ncRNA
Banliat <i>et al.</i> 2019a	Across oestrus	OF	M	Phospholipids in OF in oestrus (2 horns)
Banliat <i>et al.</i> 2020	3–5	OF, embryo (<i>in vitro</i>)	P	OF-embryo interacting proteins
Beltman <i>et al.</i> 2014	Preg: 7	UF	P	Viable vs degenerate embryo
Faulkner <i>et al.</i> 2012	Oestrus: 7	UF (plasma)	P	UF vs blood plasma proteomes
Faulkner <i>et al.</i> 2013	Oestrus: 7, 15	UF	P	Low vs high P4 effect on UF proteome
Forde <i>et al.</i> 2013	Preg: 13–19	UF (P), embryo (T)	P, (T)	Effect of P4 on gene expression
Forde <i>et al.</i> 2014a	Preg: 10–19	UF (P), embryo (T)	P, (T)	Protein changes in pregnancy
Forde <i>et al.</i> 2014b	Preg + Oestrus: 10–19	UF	M, (T)	Changes pregnant vs oestrus at different days
Forde <i>et al.</i> 2015	Preg + Oestrus: 16	UF (P), embryo (T)	P, (T)	Effect of embryo on UF composition
Forde <i>et al.</i> 2016b	Preg: 19	UF (M), embryo (T)	M, (T)	AA consumption differences by embryo gender
Forde <i>et al.</i> 2017	Preg: 19	UF (M), embryo (T)	M, (T)	Heifers, lactating and dry cows
Gegenfurtner <i>et al.</i> 2019a	Oestrus: 3	OF	P	Metabolic status, breed effects on OF proteome
Gegenfurtner <i>et al.</i> 2019b	Pregnancy: 19	UF	P	Effect of genetic merit on UF proteome
Groebner <i>et al.</i> 2011b	Oestrus: 12, 15, 18	UF	M, (T)	Changes in 41 AA through oestrus
Harlow <i>et al.</i> 2018	Oestrus: 9	UF	P	Effect of diet on UF composition
Helfrich <i>et al.</i> 2020	43–62 dpp	UF	P, (T)	Subclinical endometritis effect on UF
Hugentobler <i>et al.</i> 2007a	Oestrus: 0–14	UF, OF	M	Changes in (AA) through oestrus

(Continued)

Table 1 (Continued).

References	Collection day	Sample	Omics	Keywords
Hugentobler <i>et al.</i> 2007b	Oestrus: 0–14	OF, UF (plasma)	M	Changes in (ions) through oestrus
Hugentobler <i>et al.</i> 2008	Oestrus: 0–14	UF (plasma)	M	Glucose, lactate, pyruvate (energy)
Hugentobler <i>et al.</i> 2010	Oestrus: 3, 6	UF, OF	M	Effect of infused P4 on fluid composition
Lamy <i>et al.</i> 2016a	Across oestrus	OF	P	Side and oestrus stage effect
Lamy <i>et al.</i> 2016b	Across oestrus	OF	M	Steroids changes through oestrus
Ledgard <i>et al.</i> 2012	Oestrus: 5, 9, 14	UF	P	Asynchronic ET effect on proteome
Ledgard <i>et al.</i> 2015	15 and 42 dpp	UF	P	Subclinical endometritis vs healthy cows
Moraes <i>et al.</i> 2020a	Preg+Oestrus: 17	UF	M, (T)	Effect of preg. on UF lipid composition
Moraes <i>et al.</i> 2020b	Preg+Oestrus: 17	UF	P, M, (T)	Effect of preg. on UF proteome, metabolome
Mullen <i>et al.</i> 2012	Oestrus: 7, 13	UF	P	High fertility heifers' UF proteome
Muñoz <i>et al.</i> 2012	Preg+Oestrus: 8	UF	P	Differences in UF of <i>in vivo</i> vs AI pregnancies
Papp <i>et al.</i> 2019	Oestrus: 1,3	OF	P	Protein binding beads, OF flushing
Passaro <i>et al.</i> 2016	Preg+Oestrus: 7	UF	P	Proteome changes induced by pregnancy
Pillai <i>et al.</i> 2017	Across oestrus	OF	P	<i>Ex vivo</i> vs <i>in vitro</i> oviduct secretions
Ribeiro <i>et al.</i> 2016a	Preg+Oestrus: 15	UF (M)	M, (T)	Non-preg vs several embryo shapes
Simintiras <i>et al.</i> 2019a	Oestrus: 12, 13, 14	UF	M	Lipids (P4 suppl. effect)
Simintiras <i>et al.</i> 2019b	Oestrus: 12, 13, 14	UF	M	Nucleotides, vitamins, etc. (P4 suppl. effect)
Simintiras <i>et al.</i> 2019c	Oestrus: 12, 13, 14	UF	M	AA, carbohydrates (P4 suppl. effect)
Sponchiado <i>et al.</i> 2019	Preg+Oestrus: 7	UF	M, (T)	Embryo modulates UF metabolome
Tribulo <i>et al.</i> 2019	Oestrus: 0, 3, 5, 7	UF	M	Changes in UF metabolome in early oestrus

AA, Amino acids; AI, artificial insemination; Dpp, days post-partum; E2, oestradiol; ET, embryo transfer; EV, extracellular vesicles; FF, follicular fluid; M, metabolomics; MS, mass spectrometry; OF, oviduct fluid; P, proteomics; P4, progesterone; Preg, pregnant; T, transcriptomics; UF, uterine fluid.

In parentheses, secondary samples or omics technologies

A further consideration was raised by Velazquez *et al.* (2010) concerning chiefly *in vivo* UF sampling by flushing: due to differences in size and shape among uteri, the amount of liquid recovered varies substantially. The addition of an internal standard (a non-naturally occurring molecule such as an isotope-labelled amino acid) to the flushing medium may provide a means to correct for such differences, as reported by Vilella *et al.* (2013) in a human trial. However, this strategy has not been reported in any of the studies reviewed herein.

Two recent developments for reproductive fluid sampling deserve mention due to their increased precision. Papp *et al.* (2019) used transvaginal endoscopy to gain access to the oviduct of live animals, and sampled proteins by two methods. One was flushing using a catheter with multiple holes using, to the authors' knowledge, the only technique that allows flushing oviducts *in vivo* (Besenfelder *et al.* 2001). The other method consisted on the introduction of magnetic silica-C4 beads that bind proteins by hydrophobic adsorption. Comparisons of the two sampling methods show that both were equally robust, and that flushing resulted in one order of magnitude higher amount of protein recovered, but more proteins were identified by the C4 bead method.

There was a 45% overlap in proteins detected by both methods (Papp *et al.* 2019). Taking into consideration that beads with different binding properties could be employed to customise the proteomic fraction targeted,

the authors concluded that several approaches may be used in a complementary fashion for deeper coverage (Papp *et al.* 2019).

Helfrich *et al.* (2020) sampled UF using a device comprising a flexible Merocel (2 mm × 4 mm × 102.5 mm) sponge adhered to a plastic cannula. This was inside a working channel (stainless steel tube) with a blunt obturator to reach the uterus avoiding contamination from vagina and cervix. The sponge was rotated inside the uterine lumen for 2 min, and after retraction, proteins were extracted with PBS buffer. The constant weight of the sponge and tube and use of consistent volume of buffer allowed calculating a precise dilution factor and therefore mass of protein. This sampling technique combines low invasiveness with a reasonable sampling area (much more than by aspiration), while avoiding imprecisions due to dilution typical of flushing procedures.

The literature on the impact of sampling on pregnancy is equivocal. Berkkanoglu *et al.* (2006) reported no detrimental effect of uterine flushing immediately prior to embryo transfer in human. Martins *et al.* (2018), however, showed that bovine uteri needed 4 days to recover from the flushing disruption to return to normal pregnancy rates.

Blood contamination Furthermore, an often overlooked but highly detrimental phenomenon is the occurrence of blood contamination during sampling. This happens often (but not exclusively) during the introduc-

tion of a catheter through the vulva to collect UF or OF. Nonetheless, conspicuously few of the studies surveyed addressed or even mentioned this issue. Considering that many of the proteins UF are also found in plasma (Faulkner *et al.* 2012), determining whether relative changes are indeed the consequence of a physiological process instead of an artefact due to blood contamination may change completely the results of a trial. Although to a lesser extent, endometrial tissue is also liable to the same issue (Berendt *et al.* 2005). In this regard, Zhang *et al.* (2017) found proteins of the blood complement system in endometrial samples which they attributed to plasma contamination, possibly as a result of haemorrhage.

In some studies, a simple measure taken was to exclude samples presenting a red tinge (Martins *et al.* 2018). While this is certainly a step in the right direction, it is known that even visually transparent samples can still be contaminated (Guise & Gwazdauskas 1987). The approach taken by Helfrich *et al.* (2020) to quantify the degree of blood contamination by photometry is noteworthy. Measuring absorbance at 570 nm (haemoglobin peak) of blood and UF, they created a standard curve and thus estimated percentage of blood on UF samples. This strategy appears to assume haemoglobin occurs naturally in minute amounts in UF, although this is at odds with Faulkner *et al.* (2012), who reported haemoglobin to be more abundant in UF than blood.

A large proportion of typically intracellular proteins are found in FF (Maniwa *et al.* 2005), OF (Lamy *et al.* 2016a) and UF (Forde *et al.* 2014a). This is likely caused by a combination of (1) epithelial cell renewal and apoptosis (Mondejar *et al.* 2012), (2) damage during sampling (Papp *et al.* 2019), and (3) some of those proteins being secreted by alternative, potentially undescribed mechanisms (Gegenfurtner *et al.* 2019a). The reports of Almiñana *et al.* (2017) on the role of exosomes (small membrane-bound vesicles of endocytic origin) in oviduct-embryo communication showcasing their relevance in luminal fluids points in that direction. Supporting this are the reports of Passaro *et al.* (2016) and Harlow *et al.* (2018) that over half of the proteins detected in UF were categorised as exosomal (Gene Ontology), Banliat *et al.* (2020) also found 33 embryo-interacting proteins previously detected in oviductal exosomes along with 20 proteins secreted by non-classical pathways, and Rodrigues *et al.* (2019) reported embryo-protective effects of whole FF and FF exosomes against heat stress.

All in all, the multifaceted interface of glandular secretions, blood transudates, cellular debris and exosomes in these biofluids in physiological conditions add complexity to the task of accounting for contamination due to sampling only.

The relevance of integrating molecular research from different reproductive organs is demonstrated by

physiological research. Hunter *et al.* (2011) describe mechanisms by which FF contributes to the composition of OF, as well as the exchange of fluids between OF and UF at the utero-tubal junction. Peritoneal fluid also seems to facilitate interactions among different parts of the reproductive tract (Hunter *et al.* 2007).

Analytical platform and informatic analysis

Metabolomic studies face several hindrances, among which is the challenge in untargeted studies of interpreting mass spectrometry data and mapping it to known compounds (Patti *et al.* 2012), and in quantitative comparisons between studies, shared with other omics (reviewed by Ceciliani *et al.* 2018).

Even for compounds that are assigned a potential identity, a big hurdle is distinguishing between isomers (molecules with the same atomic composition but a different conformation), which share many physicochemical properties and are therefore challenging to separate by chromatographic and spectrometric methods (Destailats & Cruz-Hernandez 2007). Although these isomers are physically similar, they often have different biological roles (Hullin-Matsuda *et al.* 2009). Integrating ion mobility spectrometry (IMS) to the typical liquid chromatography-mass spectrometry setups has been shown to help in distinguishing fatty acids with different orientation or position of double bonds (Kyle *et al.* 2016) although this technique has not been utilised to investigate lipids in cow fertility studies.

Several articles surveyed reported absolute concentrations of metabolites. The work by Sponchiado *et al.* (2019) is unique in that they reported metabolite concentration values normalised to the respective endometrial area, in an example of absolute quantitation with an omics approach. This results from sampling and analysing UF from limited portions of the endometrium, which was performed postmortem. On a related note, a system for absolute quantification of proteins is also being developed, using a mass spectrometry function called selected reaction monitoring (SRM), combined with synthetic peptide standards of the target peptides. This targeted method allows quantification at the attomole concentration range at the cell level (Deutsch *et al.* 2019) and may represent a new standard for proteomics experiments.

Standardising measurements across studies, as seen previously, is fundamental to take full advantage of any potential biomarkers discovered. To this effect, the Livestock Metabolome Database (LMDB, <http://www.lmdb.ca>, Goldansaz *et al.* 2017) takes care precisely of this need by offering an open-access database comprising all metabolites reported from many tissues and biofluids of the five major domestic mammals (cow, sheep, goat, horse and pig). Perhaps due to the incipient state of this resource, the only component of the reproductive tract represented is FF, which is otherwise an excellent addition to omics veterinary.

Integration Most of the recent articles surveyed have presented one or more kinds of molecular integration analysis, aiding in the navigation of the myriad compounds discussed. A typical approach for gene and protein data is Gene Ontology (GO) term enrichment analysis, which delivers insight on important molecular functions, cellular components and biological processes most closely linked to the phenomenon of interest (Ashburner *et al.* 2000, The Gene Ontology Consortium 2019).

Further types of informatic inquiry are pathway and network analysis. Pathway analysis allows integration of metabolomics as well as of multi-omics data and represents a powerful tool to uncover biological processes unapparent in single omics enquiries (Li *et al.* 2013).

On that topic, to the authors' knowledge, only one study has attempted both proteomics and metabolomics analysis in a single trial in cattle. Moraes *et al.* (2020a,b) performed an exhaustive multi-omics exploration, which included secretomics (i.e. proteomics and metabolomics) of UF, coupled with transcriptomics of endometrium and conceptus. The use of pathway analysis tools supported this, but much of the integration still required a sizable amount of manual input. As multi-omics research becomes more widespread, software applications are expected to provide means of assisting with this daunting task (Pinu *et al.* 2019).

Reporting

When extracting data for this study, it was evident that the requirements for publication have become more stringent over the years; for instance, while only a few of the early studies have included raw data or the full list of compounds identified, almost all articles from 2010 onwards present either or both as supplemental information. This trend towards more transparent, reproducible research is observed in many scientific fields. The ARRIVE (Animals in Research: Reporting *In vivo* Experiments) guidelines (Kilkenny *et al.* 2010) and the REFLECT (Reporting Guidelines for Randomized Controlled Trials for Livestock and Food Safety) statement (O'Connor *et al.* 2010) have provided an excellent starting point for studies requiring experimentation on animals.

Despite said improvements, reporting of many aspects from both guidelines was incomplete or non-existent in many studies, including recent ones. This has serious implications for both comparing results across studies as well as for repeatability of the experiments described.

Despite the considerable advance apparent over the years, Winder *et al.* (2019) reached similar conclusions to ours when examining completeness of reporting in a subset of animal studies (chiefly bovine) published in a high-ranking publication for its field (Journal of Dairy

Science) in 2017, which shows that there is still a big margin for improvement.

Key molecules and processes

We performed a comparative analysis by two complementary methods. The first one was an appraisal relying on the authors' background knowledge and understanding of biochemistry and physiology. Subsequently, we augmented the reach of the first selection by text mining combined with biological database exploration, that is, pathway and network analyses (Supplementary Section 1.3).

Most articles included centre their discussion on metabolic processes. While a great deal of insight has been gained, as a result, here we examine processes that might be equally impactful on fertility but have been somewhat overlooked.

Articles studying physiological models were at first analysed separately from those focusing on endometritis (Section 2.4.4). Key molecular results were subsequently contrasted between the two classes. Figure 2 includes the molecules discussed in this section in the temporal context of early embryo development.

The most frequently mentioned metabolite groups were amino acids, carbohydrates and certain lipid classes (especially fatty acids and phospholipids). Metabolites, however, typically participate in multiple processes, which complicates generalisations regarding their specific influence on reproduction.

Conversely, the list of top proteins provided more readily understandable insight. Ranked by frequency, some of the most common proteins and protein classes related to inflammation, immune response and oxidative stress were annexins, serpins, heat shock proteins, S-100 proteins (these last two belonging to the 'acute response proteins' group), glutathione-S-transferases and tissue inhibitor of matrix metalloproteinase 2 (TIMP2). The specific proteins surveyed are discussed subsequently. The fundamental influence of those processes (both systemic and local) on reproduction appears to be somewhat disregarded in untargeted molecular research as well as in most of the articles reviewed that used healthy animals and is illustrated by the high number of molecules reported that participate in said processes.

One noteworthy exception is the work of Groebner *et al.* (2011b), which specifically addressed the molecular mechanisms of the interplay between immunity and reproduction. An important finding is that many cytokines (especially interleukin 10) are upregulated in cyclic endometrium at day 18 of oestrous cycle (Groebner *et al.* 2011b). Groebner *et al.* (2011b) also reported evidence of significant upregulation of the kynurenine pathway between days 12 and 18 of

Figure 2 Potential biomarkers of uterine suitability for pregnancy related to inflammation and oxidative stress in the cow's reproductive tract, across early pregnancy and oestrus. 'Exogenous' refers to compounds not originating from diet or the environment. AI, artificially inseminated; d, day; FF, follicular fluid; HF, highly fertile; NEFAs, non-esterified fatty acids; SF, subfertile; UF, uterine fluid. For protein and metabolite names, refer to text.

pregnancy, stimulated by interferon-tau. Both increased abundance of interleukin 10 and activation of the kynurenine pathway may be required to prevent maternal rejection of the conceptus (Shirasuna *et al.* 2012). This evidence points to an active control of inflammation and autoimmunity by the endometrium.

Gilbert (2019) reviewed the effect of disease on fertility and presented a causal chain of disease, causing inflammation and increased oxidative stress, leading to decreased fertility, although the interaction of these elements is more complex than this linear model.

Complementing the aforementioned review is the work of Guerin *et al.* (2001) which sheds light on the influence of oxidative stress on reproduction. They discuss how endogenous (especially purine metabolism and oxidative phosphorylation) and exogenous (oxygen, excess of glucose, some metallic ions, xenobiotics) factors generate reactive oxygen species (ROS) that create an inhospitable environment for oocyte and embryo development. A variety of embryo-protective mechanisms to both prevent the generation of ROS and to neutralise them have been found in cattle, human and mouse; these include internal (i.e. cellular, in the oocyte or embryo itself) and external (extracellular fluids, e.g. FF and OF). Likewise, they can be classed as enzymatic (e.g. glutathione reductase) and non-enzymatic (antioxidant molecules such as vitamins, some peptides and amino acids, etc.).

One of the less intuitive aspects of diseases (both systemic and local) is possibly the extension of their effects on reproduction well after disease resolution (Gilbert 2019). This influence does not only occur in the uterine environment, but also at the level of ovary and oocyte production (Gilbert 2019). In fact, bacterial lipopolysaccharide or endotoxin (a harmful compound produced by many microorganisms) due to infection can

be found in FF in higher concentrations than in systemic circulation or even in the infected uterus (Groebner *et al.* 2011a, Gilbert 2019).

Proteins

This section lists the most frequently mentioned proteins and protein families surveyed along with a summary of their biological significance as gathered from the relevant studies.

Annexins Annexins are a family of calcium-dependent multifunctional proteins involved, among other processes, in regulation of arachidonic acid metabolism and inhibiting activity of pro-inflammatory enzymes (Hutchinson *et al.* 2011). Ledgard *et al.* (2012) and Faulkner *et al.* (2013) found increased abundance in UF of those proteins on day 9 of oestrus compared to day 5, and day 7 of pregnancy compared to day 15 of pregnancy, respectively. Faulkner *et al.* (2013) suggest that this might be related to inhibition of phospholipase P2, an enzyme in the prostaglandin synthesis pathway (of pro-inflammatory and pro-luteolytic result). This exemplifies the temporal regulation of inflammation in early pregnancy.

Other annexins have been reported, albeit less frequently than Annexins A1 and A2. Annexin A4, for instance, was found to be upregulated in UF at day 8 pregnant compared to cyclic cows (Muñoz *et al.* 2012) and is suggested to play a similar anti-inflammatory role as Annexins A1 and A2.

Serine protease inhibitors (SERPINs) Several members of this diverse family of protease inhibitors have roles

related to inflammation and infection, and are proposed to constitute a mechanism of immunomodulation dependent on hormonal signals (Simintiras *et al.* 2019a). Some of these are potential biomarkers of different degrees of endometritis and are discussed in Section 2.4.4.

SERPINA1 was downregulated in FF of repeat breeder cows; this was hypothesised to underlie the accelerated follicular growth typically associated with lower fertility (Zachut *et al.* 2016).

In UF, SERPINA1 along with SERPINF2 and SERPIND1 were more abundant on day 17 of pregnancy in sub-fertile compared to highly fertile beef heifers, all associated to haemostasis (blood coagulation) pathways and without any reported links to fertility (Moraes *et al.* 2020a). SERPINA3 was reported as 1 of the top 20 most abundant proteins in UF of day 16 pregnant cows (Forde *et al.* 2014a) but was not detected at day 16 of oestrus in cyclic cows, although no differences were found in its transcript abundance (Forde *et al.* 2015). Many SERPINs (A3-1, A3-7, A3-8, D1 and H1) were detected in OF (Pillai *et al.* 2017, Gegenfurtner *et al.* 2019b), potentially involved in zona pellucida–sperm interactions (Cesari *et al.* 2010). The structural diversity of SERPINs entails equally varied physiological functions, both beneficial and detrimental to reproduction.

Acute response proteins: S100 and HSP (heat shock proteins) Acute response proteins are part of the early immune response, upregulated to respond to stimuli, such as infection and inflammation. Many of these have been detected in bovine UF and OF, several of which are more abundant in post-ovulation compared to pre-ovulation in OF (Lamy *et al.* 2016a).

Heat shock proteins (HSP) were first described in relation to their defensive role against heat stress but were subsequently found to be involved in protection from an array of stress factors (De Maio 1999). There is consistent reporting of upregulation of HSPs caused by pregnancy in several parts of the reproductive tract. HSPA5 was found to be more abundant in UF at day 8 of pregnancy compared to day 8 of oestrus (Muñoz *et al.* 2012) and suggested to protect the embryo from proteolytic damage (Lamy *et al.* 2016a). HSP90, HSPA6 and HSPA8 were found in higher abundance in OF in post-ovulatory compared to pre-ovulatory phase but their role is not clear (Forde *et al.* 2015); in the same work, HSP90 was more abundant at post-ovulatory phase in ipsilateral OF compared to other stages and side, and is proposed to stimulate prostaglandin E₂ secretion to regulate embryo transport.

Pregnant heifers UF contained HSPA9 (involved in embryonic attachment (Bhagwat *et al.* 2014)) and HSPD1 (annotated as a mitochondrial protein) concentrations ten-fold higher compared to cyclic heifers (Moraes *et al.* 2020a). Another protein, HSP10 was shown to be

produced by both the embryo and the endometrium (Forde *et al.* 2015).

Several studies reported HSP70 (a family comprising several proteins of molecular weight around 70 kDa, including HSPA1-14) as relevant to fertility or related conditions; due to methodological limitations, the precise protein was not always specified. Maniwa *et al.* (2005) found HSP70 to be more abundant in FF of cystic follicles, potentially decreasing the normal rates of apoptosis observed in healthy follicles. These proteins are also important in protection against oxidative stress and inflammation in UF of pregnant animals (Muñoz *et al.* 2012). Members of the HSP70 family were also detected by Massicotte *et al.* (2006) as constitutively expressed in oocytes; the authors suggested a role as a protein carrier.

Worthy of note is that in a study of exosomes and extracellular vesicles in bovine OF, numerous micro-RNAs potentially inhibiting *HSP* genes were reported by Almiñana *et al.* (2018) as varying across oestrus stages. Regulation of these proteins at different levels is likely relevant to embryo development.

S100 proteins are a family of proteins that bind calcium and tend to be overexpressed in disease, mediating inflammation and with antimicrobial activity (Lukanidin & Sleeman 2012). S100A8 and S100A9 were found to be higher in cyclic day 7 UF compared to day 13 and may modulate maternal immune function; S100A4 exhibited the opposite trend (Mullen *et al.* 2012). S100A4, in turn, was suggested to be detrimental to embryo development at day 7 of pregnancy by an altered balance between pro- and anti-inflammatory processes (Beltman *et al.* 2014). Talbot *et al.* (2010) also found S100A4 to be among the most abundant proteins in trophoblast *in vitro* cell culture.

Considering the relatively high abundance and the effect on reproduction of acute response proteins, they deserve increased attention in future molecular research.

Glutathione-S-transferases This protein family is also involved in response to oxidative stress, fulfilling a protective role in the uterus via the detoxification of ROS (Faulkner *et al.* 2013). Two glutathione-S-transferases (GSTM1 and GSTM2) were upregulated in UF on day 7 of oestrus compared to in day 15 (Faulkner *et al.* 2013). Ledgard *et al.* (2012) found that GSTM1 abundance was higher in UF in day 9 vs day 5 of oestrus. This demonstrates that reduction of oxidative stress may play a crucial part in embryo development and suggests regulation by progesterone.

Tissue inhibitor of matrix metalloproteinase 2 (TIMP2) This protein is suggested to act in limiting trophoblast invasion and possibly in endometrial remodelling (Faulkner *et al.* 2013). UF from mature cows generated by somatic cell nuclear transfer had lower TIMP2 relative abundance compared to heifers on day

14 of oestrus, but interestingly it was not detected on day 5 or 9 of oestrus in animals of either parity, and thus is hypothesised to be dependent on progesterone regulation (Ledgard *et al.* 2012). Corroborating the temporal regulation of this protein, Forde *et al.* (2014b) reported a TIMP2 increase in pregnant UF at day 16 compared to days 10 and 13 correlating with IFNT changes, and Faulkner *et al.* (2013) an increase of TIMP2 in UF at day 15 vs day 7 of oestrus. TIMP2 is considered important for successful embryo elongation (Ulbrich *et al.* 2011) as well as for regulation of protease activity during implantation (Ledgard *et al.* 2009).

In other sections of the reproductive tract TIMP2 may have detrimental effects for fertility. Zachut *et al.* (2016) found that TIMP2 was twice as abundant in FF of repeat breeder (low fertility) cows compared to control cows. They theorise that both too low or too high concentrations of this protein impede the formation of complexes with other proteins, and in this way, it is correlated to alterations in ovulation.

All in all, many proteins related to fertility differences are involved in immune, inflammatory and oxidative processes. This was understated in most of the literature surveyed. More molecular research specifically on the immune milieu of the reproductive tract is needed, in agreement with the recommendations of Ott (2019).

Metabolites

Many of the surveyed metabolomic studies used targeted approaches that look at a particular set of metabolites. This, in turn, is reflected on the frequency of metabolite classes discussed here. Even in non-targeted analyses, amino acids (AA) were also shown to be the most abundant metabolite class in UF (Tribulo *et al.* 2019).

Amino acids The amino acids most often reported as significant in the biological processes investigated were arginine, glycine, alanine and methionine.

Arginine is an amino acid with many functions related to fertility, which include being essential to produce interferon tau (IFNT) and even being an embryonic growth factor by itself, by mechanisms not fully characterised (Wang *et al.* 2015). However, Forde *et al.* (2017) found arginine in lower concentration in UF of dairy heifers compared to both lactating and dry multiparous cows, all at day 17 of pregnancy, potentially implying that high levels of arginine in UF might be detrimental to pregnancy.

Glycine and alanine have been reported as the most abundant amino acids in OF (Lamy *et al.* 2016b) and in UF (along with glutamic acid) at days 7 and 19 of pregnancy (Forde *et al.* 2017). In contrast, Tribulo *et al.* (2019) reported the most abundant AA in the first 7 days of the oestrus cycle being tryptophan, phenylalanine, tyrosine and leucine.

Glycine and alanine are both small, non-polar amino acids, which are transported by the same carrier proteins (Forde *et al.* 2014b). Glycine has been hypothesised to protect the embryo against osmotic stress and can be used by the embryo as an energy source in OF (Lamy *et al.* 2016b), and can be converted to alanine. There is conflicting evidence about the relationship between glycine concentration in UF with reproductive success: Forde *et al.* (2014b) found no effect of pregnancy on UF glycine concentrations in beef heifers from day 10 to 19 after ovulation. In contrast, Forde *et al.* (2017) reported lower abundance of both glycine and alanine in dairy heifers' UF compared to both lactating and dry mature dairy cows (all at day 19 of pregnancy), while Sponchiado *et al.* (2019) found reduced glycine concentration in UF at day 7 of pregnancy vs cyclic zebu multiparous cows.

The roles of glycine and alanine in FF are also equivocal. They have both been found to be higher in FF of lactating cows, which tend to present impaired fertility (Forde *et al.* 2016b), while other reports link higher glycine and alanine concentrations with oocytes that produce better embryos (Matoba *et al.* 2014). These differences may be a result of the effect of synchrony, follicle type, postmortem alterations, or a combination of those.

Forde *et al.* (2016b) reported that methionine concentration in FF of dairy cattle was negatively correlated with beta-hydroxybutyrate (a compound associated with negative energy balance and a detrimental effect on reproduction (Ospina *et al.* 2010)) in blood. Moreover, Sartori *et al.* (2010) hypothesised that methionine concentration in FF was relevant in fertility differences between cyclic heifers and dry cows compared to lactating animals, and this was confirmed by Forde *et al.* (2016b). As of UF, methionine presented a clear peak on day 5 compared with day 3 and day 7 in non-lactating, cyclic dairy cows (Tribulo *et al.* 2019). Methionine was also in a considerably higher concentration in pregnant compared to open beef heifers (13 times) and in highly fertile compared to sub-fertile beef heifers (18 times) at day 17 (Moraes *et al.* 2020a). Interestingly, methionine supplementation in feed was found to enhance embryo development by reducing methylation and increasing endogenous lipid reserves (Acosta *et al.* 2016).

Lipids Lipids fulfil multiple roles in reproductive processes (reviewed by Ribeiro *et al.* 2016b), and their characterisation presents specific difficulties compared to other metabolite groups. Nomenclature has improved in recent years due to initiatives such as Lipid Maps (Fahy *et al.* 2009), but technical limitations are still pervasive.

One such factor is that, due to the nature of untargeted metabolomics, only a fraction of the peaks detected can

be successfully identified. Many of those unidentified peaks are likely to correspond to lipids (Tribulo *et al.* 2019).

Arachidonic acid and eicosanoids Arachidonic acid is the precursor of a substantial number of biologically important molecules, including eicosanoids such as prostaglandins (Needleman *et al.* 1986).

The arachidonic acid content in FF was positively correlated to blood concentrations of glucose, insulin and insulin-like growth factor 1 (IGF1), and negatively correlated to beta-hydroxybutyrate at day 19 of pregnancy (Forde *et al.* 2016b). In the same study, arachidonic acid was also significantly higher in pregnant heifers and dry cows compared to lactating. Both findings indicate that arachidonic acid abundance in FF is related to a good metabolic status and might be an essential factor explaining differences in fertility linked to metabolic stress.

In UF, arachidonic acid was also more abundant in day 15 of oestrus compared to day 15 of pregnancy, likely due to a metabolic conversion to prostaglandins and other derivatives by the embryo (Forde *et al.* 2017). Similar findings by Simintiras *et al.* (2019b) support this model.

Prostaglandins, notably F2 alpha and E2, are central to different physiological processes of reproduction (Poyser 1995). UF from pregnant heifers had overall higher concentrations of PGF2a, and among those pregnant, highly fertile ones tended to have higher concentrations than sub-fertile heifers (Moraes *et al.* 2020b). The authors hypothesised that, since PGF2a is known to modulate endometrial gene expression (Spencer *et al.* 2013), related signalling pathways are important in explaining fertility differences among the two groups. Simintiras *et al.* (2019b) did not detect any prostaglandin in UF, possibly because of insufficient analytical sensitivity.

Ribeiro *et al.* (2016a) described distinct PGF2a profiles in UF of day 15 pregnant cows carrying an ovoid or filamentous embryo, over twice as high as the concentrations found for cyclic cows or pregnancies with tubular embryos at the same timepoint. These differences could be explained by either dissimilar rates of PGF2a production by the embryo across stages, of endometrial transportation or intake, or others. Some evidence also points at toxic effects of PGF2a on the oocyte (Soto *et al.* 2003) and embryo (Fazio *et al.* 1997). This is potentially related to pro-inflammatory effects (Poyser 1995) or complex temporal patterns of regulation mediated by polyunsaturated fatty acids (Caldari-Torres *et al.* 2006). Roles of fatty acids on reproductive physiology are discussed subsequently.

Fatty acids Findings in correlations between fatty acid abundance to reproductive success are complex to integrate but point in a similar direction. Lower concentrations of (omega-3) polyunsaturated fatty acids, particularly alpha-linolenic acid (ALA) and

docosahexanoic acid (DHA), were found in FF of lactating cows (of well-known reduced fertility (Forde *et al.* 2017)) compared to heifers and non-lactating cows (Forde *et al.* 2016b). Docosahexanoic acid was also in lower concentration in FF of lactating cows compared to heifers by Bender *et al.* (2010). Supplementation of physiological concentrations (1 μ M) of DHA improved bovine oocyte quality *in vitro*, possibly by aiding cytoplasm maturation of oocytes (Oseikria *et al.* 2016). Alpha-linolenic acid is vital for oocyte maturation through the MAPK pathway as well as through PGE2 synthesis, and its supplementation improved *in vitro* maturation of oocytes resulting from cumulus-oocyte complexes (Marei *et al.* 2009). Similarly, Matoba *et al.* (2014) found that the probability of oocytes to produce embryos that reach blastocyst stage was positively correlated to ALA concentration in FF and negatively correlated to the concentration of total saturated fatty acids, especially palmitic acid.

Palmitic and stearic acids are major components of the bovine FF (Leroy *et al.* 2005, Bender *et al.* 2010) and have been reported to be negatively associated with embryo quality (Leroy *et al.* 2005). Both palmitic and stearic acid have also been shown to induce apoptosis in human granulosa cells *in vitro* by affecting acyl-coA metabolism, and arachidonic acid supplementation countered these detrimental effects (Mu *et al.* 2001).

Phospholipids Phospholipid profiles varied considerably in endometrium but not in oviduct tissue at days 4 and 7 of oestrus in a study by Belaz *et al.* (2016), suggested to depend on follicle size and endocrine conditions. Sponchiado *et al.* (2019) showed that exposure to a day 7 embryo caused a general decrease in concentration of phospholipids in UF, which they attribute to increased cell membrane synthesis.

Moraes *et al.* (2020b) found that several phosphatidylcholines (typical components of cell membranes) were significantly more abundant in the pregnant uterine lumen at day 17, potentially due to the secretion of EV by the elongating conceptus. Polyunsaturated phosphatidylcholine appears to significantly attenuate the damaging, pro-inflammatory effects of LPS injection on murine organs (Jung *et al.* 2013) and might fulfil similar protective functions in the bovine UF.

Other metabolites Glucose is an indispensable nutrient for pre-implantation in cow, at least from the blastocyst stage (Krisner & Prather 2012). Simintiras *et al.* (2019c) found an increase of UF glucose at day 14 of oestrus caused by progesterone supplementation, and stronger increasing trends in the concentration of mannitol at days 12–14, also heightened by progesterone. The authors propose that elevated glucose transport towards UF and its conversion to mannitol by aldose reductase are relevant to embryo elongation. Moreover, Moraes *et al.* (2020b) reported glucose to be signifi-

cantly more abundant in UF at day 17 of pregnancy compared to oestrus; they make a case for glucose availability and glucose transporters' expression in embryo to be crucial for its development.

Carnitines and derivatives have been found in increased abundance in day 17 UF of pregnant compared to cyclic cows, as well as in highly fertile compared to sub-fertile cows at the same time of pregnancy, likely to fulfil fatty acid transport or antioxidant functions (Moraes *et al.* 2020a).

In summary, the wealth in metabolomics data amassed so far has increased our understanding of reproduction. However, we suggest that further integration and improvements in analysis tools are needed, especially supporting more sophisticated analytical platforms, such as multidimensional chromatography coupled to mass spectrometry (Stanstrup *et al.* 2019) and ion mobility spectrometry.

Main themes

The surveyed body of research studied numerous aspects related to reproductive health and performance though less about immune response even though many proteins identified are immune related. A selection of the articles' findings is discussed here mainly according to their direct relevance to fertility, and secondly to how their findings aid towards biomarker discovery.

Based on the goal, type of the experiment and the phenomenon studied, we grouped most of them into the themes of biomarkers for fertility, hormones and differences between cyclic and pregnant cows.

Some articles were relevant to more than one theme and are therefore discussed more than once. Other studies provided limited value in terms of reliable biochemical information (especially the earliest articles) and were not discussed in the text.

Potential biomarker candidates of fertility

The word 'biomarker' is used liberally in molecular biology research and the same is apparent in this selection of studies. Differentially expressed proteins or other molecules should be initially reported as potential biomarker candidates. To qualify as a proper biomarker, the molecule or set of molecules needs first be validated in greater numbers and in different sets of samples and by using various methodologies and parameters (e.g. ELISA, Western blot, MRM) (Martins-de-Souza 2010).

Having made that important distinction, the studies in this section report compounds (or molecular features) proposed to affect fertility directly. Articles in Sections 2.4.2 and 2.4.3 typically share the technical approach with these, however their biological question is related to reproductive function in a more indirect fashion.

Molecular imaging technology over a tissue section is a novel technology applied to biomarker discovery. Gonçalves *et al.* (2016) evaluated embryo development stage by mass spectrometry imaging, showing that metabolic fingerprinting was powerful enough to allow discrimination of five developmental stages: oocytes, 2-, 4-, and 8-cell embryos, and blastocysts. By further tandem mass spectrometry (MS/MS) of selected features, seven molecules of different chemical groups were suggested as potential biomarker candidates, most notably phospholipids.

The same group analysed the metabolite composition of FF to determine differences between animals of different reproductive potential (Guerreiro *et al.* 2018). They developed a seven-compound model that accurately classified non-lactating *Bos indicus* in three fertility groups according to the number of oocytes they produced (*Fert+*, *control* and *Fert-*). This classification is based on evidence that a higher number of oocytes can be an important factor for fertility in the production of embryos *in vitro* (Pontes *et al.* 2011). Some clear trends emerged from the compounds used for prediction: the FF of animals producing few oocytes presented augmented inflammatory compounds, whereas FF of cows producing a higher number of oocytes showed increased phenolic and flavonoid metabolites (notably resveratrol and derivatives), compounds with well-known antioxidant properties (Mikulski & Molski 2010). The authors discuss the implications on the reproductive function of these compounds in the pasture the animals were fed, although the intricacies of the interplay between inflammatory and antioxidant processes remain undescribed (Guerreiro *et al.* 2018).

One research group performed methodologically complementary experiments examining the early embryo proteome. Taken together, the two works (Deutsch *et al.* 2014, Demant *et al.* 2015) described proteome signatures encompassing the whole range of oocyte to blastocyst. Specifically, Deutsch *et al.* (2014) studied oocytes, zygotes, 2- and 4-cell embryos, finding 87 differentially abundant proteins between the four stages; a panel of five selected proteins allowed discrimination. In turn, Demant *et al.* (2015) reported a sizable increase in abundance of proteins (73% of the total) from day 5 morulae to day 7 blastocyst stages. Interestingly, in agreement with the conclusions of Talbot *et al.* (2010), the authors emphasised the importance of several annexins in the transition from morula to blastocyst. Worthy of note is that each study carried out validation by an independent technique, either saturation 2-DIGE or SRM (selected reaction monitoring using mass spectrometry, discussed previously), strengthening the reliability of their results. A further necessary step would be to test the predicting value of their potential biomarker candidates on an independent dataset.

Effect of hormonal differences

A nexus between metabolism and reproduction is hormonal regulation; the full significance of this interplay and all its components are yet to be understood, although an indisputable main actor is progesterone (Diskin & Morris 2008). The effect of differences in progesterone levels on the proteome and metabolome of reproductive tissues was evident in all the studies surveyed, both naturally occurring and the following supplementation. Simintiras *et al.* (2019a,b,c) found supplementation with progesterone from day 3 of oestrus caused clear changes in metabolites of all classes examined at days 12, 13 and 14 of oestrus cycle in crossbred beef heifers (lipids, amino acids, carbohydrates, peptides, energy substrates, xenobiotics, nucleotides, vitamins and co-factors).

Changes in FF composition throughout oestrus were studied in a foundational study by Wise (1987). He measured ionic, hormonal and enzymatic parameters in both large and small follicles from 152 heifers, reporting reduced enzymatic activity, as well as decreased Ca and increased Mg concentrations in larger follicles. Similar results for enzymatic activity were described by Aller *et al.* (2013). Orsi *et al.* (2005) reported an effect of day of oestrus cycle on both the abundance of energy substrates (pyruvate, lactate and glucose) and most amino acids. They also compared the composition of FF with that of oocyte maturation medium, suggesting that the sizable difference in pyruvate to lactate ratio may be an indicator of further differences on oocyte metabolism.

Lamy *et al.* (2016a,b) found clear fluctuations in proteins, steroid hormones and metabolites in OF throughout the oestrus cycle, whereas in FF differences were less pronounced (Orsi *et al.* 2005).

A better understanding of the interactions between molecules (proteins and metabolites) in the reproductive tract will lead to deeper insight on the needs of the embryo. This will aid in the improvement of *in vitro* embryo production and embryo selection for transfer.

We anticipate effective diagnostic and prognostic applications of biomarkers (and perhaps complementary to blood-based) within the reproductive tract following the rapid progress of research in this area.

Differences between pregnancy and cyclic status

The composition of UF is regulated by both endocrine mechanisms independent of the presence of an embryo (Forde *et al.* 2014a, Tribulo *et al.* 2019), and the paracrine influence of an embryo (Sponchiado *et al.* 2017). A first stage of embryo-maternal communication in uterus has been shown to commence at least as early as day 7 after conception (Sponchiado *et al.* 2017), while a second stage is triggered by interferon tau signalling at around day 14 of pregnancy (Robinson *et al.* 2006).

Forde *et al.* (2011) and Passaro *et al.* (2016) found no differences between cyclic and pregnant UF in day

7 proteome, and in days 5, 7 and 13 transcriptome, respectively. Sponchiado *et al.* (2017), however, showed distinct endometrial expression changes induced by pregnancy in the utero-tubal junction and the anterior area, but not in the posterior area of the endometrium, all at day 7. Thus, it is likely that sensitivity limitations of sampling techniques and analytical equipment have prevented many studies from characterising very localised and potentially weaker molecular changes caused by the early embryo in the first week of pregnancy (Gomez & Munoz 2015). The same study also suggests that a substantial part the embryo's paracrine effect is likely mediated primarily by post-translational modifications of proteins and micro RNA exchanges through exosomes and EVs, as these are more dynamic and versatile mechanisms compared to *de novo* synthesis of mRNA. In support of a multifactor model for embryo-maternal interaction, Nakamura *et al.* (2020) showed that EVs isolated from day 17 pregnant uterus induced a pro-inflammatory response in endometrium cell culture independent of IFNT; this is hypothesised to regulate uterine receptivity to facilitate conceptus implantation.

Deutsch *et al.* (2019) integrated proteomics data from six studies through a GO (Gene Ontology) meta-analysis, identifying several significant process clusters in the pre-implantation period of pregnancy, namely 'MHC class II antigen presentation', 'degradation of the extracellular matrix', 'platelet degranulation', 'activation of chaperones by IRE1alpha' and 'glycosphingolipid metabolism'. Each of those clusters contains both up- and downregulated proteins, with limited overlap across studies (cathelicidins, legumain, aldose reductase, and TIMP2). The absence of clear-cut trends complicates shortlisting molecules with high biomarker potential for reproductive success.

Endometritis

Endometritis is an infection of the uterine endometrium with a serious negative effect on cow health and fertility (McDougall 2001). The molecular characterisation of local response to infection in reproductive tissues provides an extreme example of detrimental conditions for fertility to which milder conditions might be related to. Four studies investigating clinical (Choe *et al.* 2010, Zhang *et al.* 2017) and subclinical (Ledgard *et al.* 2015, Helfrich *et al.* 2020) endometritis have been surveyed in this systematic review. Conjunctly, they highlight the importance of inflammation-related proteins, especially acute phase proteins; among those are heat shock proteins (HSP) and Major Histocompatibility Complex 1 (MHC1) (Choe *et al.* 2010) as well as cathelicidins, of antimicrobial function (Kosciuczuk *et al.* 2012). Zhang *et al.* (2017) found five (cathelicidins 2, 3, 5–7) to be significantly more abundant in endometritic tissue. Ledgard *et al.* (2015) found cathelicidins highly correlated with the percentage of polymorphonuclear

leukocytes in UF affected by subclinical endometritis, indicating their production by these immune cells.

Several cytokines are intimately involved in the aetiology of endometritis. Notably, [Helfrich et al. \(2020\)](#) found that pro-inflammatory interleukins 1B, 8 and 17A in UF are potential biomarkers of cytological endometritis. Conversely, [Choe et al. \(2010\)](#) found another pro-inflammatory interleukin (IL2) only in healthy endometrial tissue. Transcription of IL10, an anti-inflammatory cytokine ([Siewe et al. 2006](#)), was upregulated in subclinical endometritic tissue [Helfrich et al. \(2020\)](#) but no changes were detected at the protein level. On the contrary, the protein was found in higher abundance in clinically endometritic UF by [Kim et al. \(2014\)](#). These discrepancies might be related to the degree of pathology or by post-transcriptional regulation.

[Ledgard et al. \(2015\)](#) found several serine protease inhibitors (SERPINB1,3,4) upregulated in UF during subclinical endometritis and speculated about their role in regulating immune homeostasis and inflammation. SERPINA10 was upregulated in endometrium with endometritis, possibly with a similar role as the above ([Zhang et al. 2017](#)). Both works also describe an upregulation of annexins, most notably ANXA1 and ANXA2, potentially modulating inflammation through inhibition of PGF2a synthesis or several pro-inflammatory enzymes ([Hutchinson et al. 2011](#)).

S100 proteins have also been characterised related to endometritis. S100A9 was also increased in endometritic UF according to [Ledgard et al. \(2015\)](#) but [Helfrich et al. \(2020\)](#) found no difference in the transcript levels of this protein in endometrium of healthy and diseased animals. This might be associated to regulatory mechanisms posterior to transcription and caused by differences in number of days post-partum or genetic differences between the animal models used. Another protein of potent microbial activity, S100A12, was significantly more abundant in inflamed endometrium ([Zhang et al. 2017](#)). These protein families are further discussed in Section 3.3.1.

Immunoglobulins (Ig) are another important protein family related to immune processes, with proven protective roles against pathogens reviewed by [Dhaliwal et al. \(2001\)](#). IgG1 was found to be one of the 20 most abundant proteins in pregnant UF ([Forde et al. 2014a](#)). [Alavi-Shoushtari et al. \(2006\)](#) measured several of these proteins in UF in a targeted approach. They highlighted an increase of IgA1 in the follicular phase in agreement with the enhanced uterus defence during that phase compared to the luteal phase. Surprisingly, [Zhang et al. \(2017\)](#) described a decrease in the abundance of several immunoglobulins (IgA1, IgV3–6, IgG4) in diseased compared to healthy endometrial tissue. This could be related to an increased transport from endometrium to UF ([Dhaliwal et al. 2001](#)).

In summary, this section discussed several molecular processes underpinning the inflammation and altered

immune response resulting from clinical and subclinical endometritis. Some of those mechanisms resemble those underlying reproductive failure in physiologically normal animals. Thus, they are worth researching further to better understand pregnancy failures without a clear aetiology.

Further research

The interplay between endocrine, metabolic and pathological processes necessitates further investigation, especially to elucidate any possible use of diet adjustments to 'tailor' the molecular milieu of the reproductive tract. The remarkable work of [Harlow et al. \(2018\)](#) examining the effect of diet composition on the composition of bovine UF opens a new area to ameliorate fertility problems in cattle and potentially other species. Also, combining this with embryo transfer can help distinguish the effects of oocyte quality (i.e. supplementation across months before pregnancy) from the immediate effect of the maternal reproductive tract (supplementation around the time of pregnancy) on embryo development.

As discussed previously, characterising the early embryo milieu is difficult because a portion of the embryo-maternal interactions is highly localised and thus challenging to measure. Two research models are particularly promising to cope with this: multiple embryo transfer ([Gomez & Munoz 2015](#)) and localised flushing *in vivo*.

Multiple embryo transfer ($n > 20$) provides a means to amplify early embryo signals and has been shown not to affect the hormonal environment in non-physiological ways ([Gomez & Munoz 2015](#)). Further proof of the feasibility of this model is the work of [Maillo et al. \(2015\)](#) in which an embryo effect on oviductal transcriptome at day 3 after ovulation was detected only when 50 zygotes were transferred to the oviduct 1.5 days earlier; no effect was detected in artificially inseminated animals carrying a single embryo.

On the other hand, using single embryo research models is more biologically reliable, but as mentioned the insufficient technical sensitivity is a major drawback. The answer may lie in combining and refining recent developments: a localised sampling technique based on clamping ([Sponchiado et al. 2017, 2019](#)) or transvaginal endoscopy ([Papp et al. 2019](#)) with a tool such as Merocel sponges ([Helfrich et al. 2020](#)) to be applied *in vivo*, thus preventing post mortem-induced alterations. To the authors' knowledge, these techniques have not been applied conjunctly to date but present enormous potential.

Antioxidant supplementation in feed is an attractive possibility, though results have been inconsistent (reviewed by [Hansen 2010](#)), highlighting the need for a deeper understanding of the interplay between oxidative status and reproduction, especially within the reproductive tract. Some promising results in mice and bovine embryos produced *in vitro* have been obtained

by use of flavonoids and anthocyanins (reviewed by Hansen 2010), or N-acetyl cysteine, although its effect is inconclusive (Bromfield & Iacovides 2017).

Guerreiro *et al.* (2018) found several antioxidant molecules in FF of high fertility zebu cows originating from pasture on which the animals fed (*Brachiaria* spp.). One of those, resveratrol, has been shown to improve *in vitro* fertilisation outcome (Takeo *et al.* 2014) and blastocyst development (Salzano *et al.* 2014) when added to oocyte maturation medium. It is unclear what underlies the differences in ingestion or absorption of these compounds and how it relates to the animals' reproductive capacity. However, feed supplementation of resveratrol-containing waste materials or by-products such as grape skin (Pascual-Marti *et al.* 2001) is an interesting possibility to explore.

As an additional consideration, systemic research at the levels of plasma and reproductive tract as surveyed in the present review represent a step in the right direction. However, this has not fully delivered, to a degree because of difficulties inherent to plasma (especially dominance of a few proteins (Faulkner *et al.* 2011)). To achieve a deeper understanding of molecular aspects of fertility at the systemic level, two papers are exemplary of approaches to follow: Sun *et al.* (2015) determined the effect of two types of feed on the metabolome of four different biofluids (rumen, milk, serum and urine), and Moore *et al.* (2019) studied the influence of the bacterial metagenome of early post-partum bovine endometrium on endometrial transcriptome.

Conclusions

Cattle reproductive research by proteomics and metabolomics is a relatively young field that has made substantial progress in terms of the volume and quality of the information. Based on the surveyed articles, the link between disease, oxidative stress, inflammation and immune response to short- and long-term fertility has received some coverage but necessitates further molecular research.

On a different note, the need for standards to refer to when comparing pieces of research can be realised by systematising animal management specifications, while improving and implementing techniques for absolute quantification of molecules. Additionally, following guidelines such as REFLECT and ARRIVE will ensure a much higher standard of publication quality with only limited extra effort required.

Two promising research avenues are systems-biology investigation of the effect of supplementing specific compounds (methionine, resveratrol) on the molecular milieu of the reproductive tract, and the further development of highly localised, *in vivo* sampling techniques. We envision a breakthrough in the field within the next few years as omics analysis equipment becomes more affordable and widespread,

and software tools easier to use. Any further advances may benefit directly the bovine agricultural sector with improvements to fertility, and potentially help alleviate fertility problems in other mammal species.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0047>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by a partnership (DRCX1302) between New Zealand dairy farmers through DairyNZ Inc. and the Ministry of Business, Innovation, and Employment (MBIE), and by AgResearch Ltd. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contribution statement

N A was responsible for the original manuscript. N A and J L G were responsible for investigation and formal analysis. D K B, J L G and J D M provided supervision and crucial intellectual input into the design, writing and approval of this work.

Acknowledgements

The authors wish to acknowledge the gracious support of Jenny Juengel by revising the draft, providing valuable comments and criticism. They also wish to thank Claudine Pausma for the artwork.

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Received 30 January 2020

First decision 9 March 2020

Revised manuscript received 19 June 2020

Accepted 3 August 2020