

**Investigation of equine fertility mechanisms
through proteomic analysis of stallion
spermatozoa, early equine embryos and
pregnant mare uterine fluid**

Aleona Swegen

B Veterinary Science

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Declarations

Originality

I hereby certify that to the best of my knowledge and belief this thesis is my own work and contains no material previously published or written by another person except where due references and acknowledgements are made. It contains no material which has been previously submitted by me for the award of any other degree or diploma in any university or other tertiary institution.

Thesis by Publication

I hereby certify that this thesis is in the form of a series of papers. I have included as part of the thesis a written statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

Signed

Aleona Swegen

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Some say our student office is 'cramped', but I like to call it vibrant – and wouldn't have it any other way! I have been so lucky to share the last four years with a fantastic group of friends as fellow grad students – special thanks to Liz, Jacinta and Brendan for the laughs, coffee, cats, yoga, and consistently excellent company.

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This thesis was written and researched on Awabakal lands.

Publications included in this thesis

Chapter 1:

Swegen, A., & Aitken, R. J. (2014). **Prospects for immunocontraception in feral horse population control: exploring novel targets for an equine fertility vaccine.** *Reproduction, Fertility & Development*. doi: 10.1071/rd14280 [published]

Chapter 2:

Swegen, A., Curry, B. J., Gibb, Z., Lambourne, S. R., Smith, N. D., & Aitken, R. J. (2015). **Investigation of the stallion sperm proteome by mass spectrometry.** *Reproduction*, 149(3), 235-244. doi: 10.1530/rep-14-0500 [published]

Chapter 3:

Swegen, A., Lambourne, S. R., Aitken, R. J., & Gibb, Z. (2016). **Rosiglitazone Improves Stallion Sperm Motility, ATP Content, and Mitochondrial Function.** *Biology of Reproduction*. doi: 10.1095/biolreprod.116.142687 [accepted/published online early]

Chapter 4:

Swegen, A., Grupen, C. G., Gibb, Z., Baker, M., de Ruijter-Villani, M., Smith, N. D., Stout, T. A. E., Aitken, R. J. **From peptide masses to pregnancy maintenance: A comprehensive proteomic analysis of the early equine embryonic secretome and pregnant mare uterine fluid.** [submitted/under review]

Conference presentations

Swegen A, Gibb Z, Aitken RJ, “Metabolic flexibility of stallion spermatozoa: improving sperm storage strategies with rosiglitazone” (2016) Oral presentation, Society for Reproductive Biology Annual Conference, Gold Coast, QLD, Australia.

Swegen A, Grupen CG, Gibb Z, De Iuliis G, Smith ND, Tazawa SP, Aitken RJ, “Novel prostaglandin regulating mechanisms revealed by proteomic analysis of early equine pregnancy” (2016) Oral and poster presentations, International Congress on Animal Reproduction, Tours, France.

Swegen A, Gibb Z, Aitken RJ, “Relationships between disease and fertility: 4-Hydroxynonenal interferes with AMP-kinase activation in stallion spermatozoa” (2015). Poster presentation, 48th Annual Meeting of the Society for the Study of Reproduction, San Juan, Puerto Rico.

Swegen A, Aitken RJ, “Fatty acid metabolism contributes to energy production in stallion spermatozoa” (2014). Poster presentation, 12th International Symposium on Spermatology, Newcastle, NSW.

Swegen A, Aitken RJ, “Stallion spermatozoa and AMP-activated protein kinase: Can we make sperm hibernate?” (2014). Oral presentation, 12th International Symposium on Spermatology, Newcastle, NSW.

Swegen A, Aitken RJ, “AICAR improves motility of stallion spermatozoa in vitro” (2014). Poster presentation, Association for Applied Animal Andrology 9th Biennial Conference, Newcastle, NSW.

Swegen A, Aitken RJ, “Characterisation of the stallion sperm proteome” (2014). Oral presentation, International Symposium on Equine Reproduction XI, Hamilton, New Zealand. Peer-reviewed; published in Journal of Equine Veterinary Science, Volume 34, Issue 1, 35-37.

Abstract

Equine breeding industries suffer from many inefficiencies arising from deficits in our understanding of reproductive physiology in this species. This includes cases of idiopathic infertility and suboptimal resistance of spermatozoa to chilled storage and cryopreservation in stallions, and high rates of early embryo loss in mares. At the same time, unchecked breeding in populations of feral horses, an introduced species in the Australian landscape, contributes to damage of sensitive ecosystems and poses an ethical conundrum in light of controversy surrounding lethal population control methods. Existing fertility control agents are lacking in their efficacy and specificity. Improving our understanding of the molecular mechanisms underlying reproductive function in horses is paramount if we are to achieve better outcomes in breeding industries and develop novel contraceptive strategies for feral horses. The advent of highly sensitive mass spectrometry technologies provides a distinct opportunity to characterize the proteomic profiles of the major phenotypic elements of equine reproduction – the spermatozoon and the early embryo.

This thesis encompasses a series of studies whose aims were 1) to utilize cutting edge mass spectrometry technology to characterize the proteomes of stallion spermatozoa and of early equine embryos together with their secretome and immediate uterine environment, 2) to integrate proteomic data with bioinformatics and in vitro experimentation and thus elicit an improved understanding of equine reproductive function and 3) to deliver applied outcomes in fertility manipulation (including contraceptive targets and strategies for improving fertility).

Mass spectrometry driven proteomic analysis of equine spermatozoa revealed 1030 proteins. Together with gene ontology analyses these provided new information about the metabolism, antioxidant defenses and receptors of stallion spermatozoa. Mitochondrial proteins and those involved in catabolic processes constituted dominant categories. Several enzymes specific to beta-oxidation of fatty acids were identified, and further in vitro experiments demonstrated that beta-oxidation contributes to metabolism and motility in stallion spermatozoa.

Identification of a series of receptors, kinases and metabolic enzymes in the sperm proteome led to an investigation of the roles of peroxisome proliferator activating receptor gamma (PPARG) and the metabolic switch, AMP-activated protein kinase (AMPK), in regulation of stallion sperm metabolism and in mediating the remarkable effects of an anti-diabetic pharmaceutical, rosiglitazone, on sperm motility, reactive oxygen species production, mitochondrial function and ATP production. The characterization of functional AMPK in stallion spermatozoa provided a novel avenue for the manipulation of sperm metabolism and improvement of sperm storage methods. Accordingly, rosiglitazone substantially alleviated the time-dependent deterioration of stallion spermatozoa during ambient temperature storage by diverting metabolism away from oxidative phosphorylation and towards glycolysis, with novel implications for preservation of stallion spermatozoa.

In the final study, we present the first successful proteomic analysis of several components of the equine pregnancy: proteins secreted by early embryos, blastocoel fluid composition, the embryonic capsule, and uterine luminal fluid proteins. Integrating these findings with existing literature yields novel insights into how these proteins fit into the complex puzzle of pregnancy establishment and maintenance.

The studies presented herewith demonstrate the successful utilization of cutting edge mass spectrometric technology for comprehensive analysis of the proteins that define the male gamete in the stallion and early pregnancy in the mare, with implications for both equine fertility control and fertility enhancement.

Aims and overview

The broad aim of this thesis is to harness cutting edge proteomic technologies to further our understanding of the mechanisms underlying equine fertility. The work presented here represents part of a larger project initiated upon partnership with representatives of the equine breeding industries of the Hunter region, specifically Harness Racing Australia and the Hunter Valley Equine Research Foundation, under the umbrella of an Australian Research Council Industry Linkage Grant. Drawing together one of the world's most prominent horse breeding regions and a Priority Research Centre for reproductive science, the project sought to understand and improve the fertility of stallions at stud, to elicit robust clinical fertility assessment methods, and to develop novel sperm storage strategies. Early on in the stallion fertility journey we found ourselves asking questions about whether various concepts are relevant to stallion spermatozoa, or if these cells possess a given enzyme, or if there is any evidence spermatozoa are capable of specific metabolic processes, and so on. It soon became clear we needed a comprehensive proteomic resource that would provide a reference point in beginning to answer functional questions about the male gamete in this species, and would serve as a foundation stone for further studies. Thus, work began on mapping the stallion sperm proteome that would ultimately result in "Investigation of the stallion sperm proteome by mass spectrometry", included as Chapter 2 of this thesis. Another key partnership was soon established, between our lab and the Invasive Animals Cooperative Research Centre (IA CRC). With feral horse overpopulation a rapidly growing and highly controversial concern in Australia, the IA CRC sought to foster collaborative research in order to develop new strategies for invasive animal fertility control. Thus the aims of the doctoral project pertaining to this thesis expanded to encompass the need for equine fertility control, offering the unique opportunity to apply our research to both sides of equine fertility regulation – enhancement *and* inhibition of reproductive function.

Chapter 1 explores how we can exploit the many quirks of reproductive biology unique to the horse in developing completely novel strategies to tackle feral horse overpopulation, moving away from the focus on incremental improvements within existing immunocontraceptive vaccines. Here, a review of the current literature illuminated an urgent need for characterization of the protein components not only of spermatozoa, but

also the principal components of equine pregnancy. Proteomic analysis of the early pregnancy milieu, including embryonic secretions and uterine luminal fluid, in addition to the sperm proteome, would begin to answer the fundamental question at the core of developing a species-specific fertility agent: What are the antigenic, functional proteins that could be targeted with a new fertility-inhibiting vaccine or toxin? To this end, fruitful collaborations with colleagues at the University of Sydney and Utrecht University yielded the first comprehensive analysis of proteins secreted by the equine embryo and the pregnant mare endometrium around the time of maternal recognition of pregnancy, documented in Chapter 4 of this thesis.

Both the sperm proteome and pregnancy proteome studies led to significant advances in our understanding of equine reproductive physiology, with respect to individual proteins combined followed up by investigation of their function, and entire groupings of biological processes revealed by gene ontology analyses. The unexpected role of beta-oxidation of fatty acids in sperm function is documented together with the proteome in Chapter 2. Similarly, the work presented in Chapter 3 manifested as a direct sequel to the sperm proteome, flowing on from the initial identification of an array of receptors, kinases and metabolic enzymes. Here we dissect the functional significance of a receptor and a kinase, and the role they play in the spermatozoon's remarkable response to the anti-diabetic pharmaceutical, rosiglitazone. This study illustrates one example of an applied outcome stemming from the sperm proteome project: the development of an ambient temperature sperm storage medium that succeeds in maintaining adequate sperm quality for at least 6 days.

Together, the studies presented in this thesis seek to improve our understanding of equine fertility mechanisms, including sperm metabolism and embryo-maternal interactions. We achieve this through engaging cutting edge mass spectrometry to characterize the proteomes of stallion spermatozoa and of early equine embryos together with their secretome and immediate uterine environment. These analyses are further supported by integration of proteomic data with bioinformatics and in vitro experimentation, culminating in applied outcomes for manipulation of equine fertility.

By signing below I confirm that the PhD candidate Aleona Swegen has contributed upward of 50% towards the data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

R John Aitken

Date:

Zamira Gibb

Date:

Benjamin J Curry

Date:

Sarah R Lambourne

Date:

Nathan D Smith

Date:

Mark Baker

Date:

Christopher G Grupen

Date:

Marta de Ruijter-Villani

Date:

Tom A E Stout

Date:

Francis Martin (FADRT)

Date: