TOWARDS NON-SURGICAL STERILISATION OF FERAL HORSES: Biomarkers of folliculogenesis and covalent modification of proteins using electrophilic aldehydes

Sally Elizabeth Hall

Bachelor of Animal and Veterinary Bioscience

(Honours)

PhD in Biological Sciences

27 January 2017

Thesis submitted to the Faculty of Science and Information Technology, The University of Newcastle, Australia in fulfilment of the requirement of the degree of the Doctor of Philosophy

THE UNIVERSITY OF
NEWCASTLE
AUSTRALIA

Declarations

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and does not contain any material which has been submitted previously for any degree or diploma to any University or Institution.

Statement of Authorship

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

Sally Hall

Bachelor of Animal and Veterinary Bioscience (Honours)

Acknowledgements

There are many people I would like to thank for encouraging me on the PhD journey. Over the last four years, I have learnt more about myself than I anticipated. I experienced significant life-altering challenges, and I guess what really took me by surprise was that life did not stop while I was doing the PhD. There were still personal hardships I had to face alone, testing my will to keep moving forward, and asking me how much I want this. Having now experienced the pressures of a PhD I've come to believe it is a true measure of resilience. You never stop being tested.

I am so grateful to be a part of the Invasive Animals CRC group. I loved spending time at the camps, getting to know everyone, and realising that we are all having similar setbacks and challenges. I will always have fond memories of our musical improv by the campfire. Tony, thank you for always being the rock for the IACRC students. It has been an honour studying as part of IACRC, and the additional support that the students receive explains why we have such a high completion rate.

Jessie, thank you so much for your friendship. You taught this animal science girl the ins and outs of molecular biology! I had so much fun with you when I first began my PhD, and it has been such a joy spending more time with you again this year. Thank you for guiding me in the lab and being someone that I can always come to for advice.

Brett, our first real conversation was such a pivotal one for me. From day one you supported me and I slowly regained some of the confidence that I had lost during the start of my candidature. You are the epitome of academic excellence, scrupulously hardworking, and supportive of your students. You pushed me harder than my other supervisors to do better, and it has been an interesting thing to reflect on. I really value that you did that for me because it made me go that extra mile to

create a beautiful and cohesive story. It has been a pleasure being guided by you and a joy to see you move from strength to strength into your fellowship and professorship.

Zamira, it was only when we started working together that I finally saw my PhD taking real form. Something had been missing before; maybe it was sperm, maybe it was you. Your support and friendship has been hilarious fun, and I am so lucky that I could always come to you and feel understood. I'm sure other students won't ever get to experience that. It was very comforting to look up to you and know that a fellow AVBS kid had stepped into the biotechnology field and made it. Thanks for always making funny faces at me when walking past each other, always coercing my face into a smile no matter how I was feeling that day.

John, every word that comes out of your mouth is poetic and inspiring. There have been many moments in focus group when I've wished I could record the light bulb moments that you share with us, for fear I won't be able to get it all down on paper in time. You are an artist in this field, and that is what makes you a leader. The little words of encouragement you have shared with me have been the reason I have known that I could do this. "A PhD is a third hard work, a third intelligence, and a third show business. Sally, you've got the show business." You are a compassionate and gentle person, and as someone who has turned out to be very sensitive, I needed you as my supervisor. Thank you for taking on the challenge.

To my fellow PhD friends, I'm so glad we got to go on this ride together. Jacinta, you are so generous and caring. I am so grateful for everything you have done for me while I have lived in Newcastle. Thank you for drawing beautiful henna patterns on me, and always reaching out no matter how distanced I was at times. Brendan, we only really got our mojo once you started sitting next to me, but damn, you have made me laugh more than anyone else in a long time. Half the time you drove me crazy, and that allowed me to realise that I need to chill out and just let

myself be a human being and have fun. I'm really going to miss talking to you in pig Latin but won't miss my hair bun attacks (you know what I'm talking about). Aleona, I'm so grateful for the IACRC camps and ICAR because those events were such a pivotal part in us getting to know each other and becoming close friends. Thank you for always lending an ear and giving me advice when I need to talk something out, or when it's a bit bigger than that and I really need company. You are an incredible friend.

To my parents, Heather and Chris, thank you for always being supportive of me no matter how irrational my goals are. You always let me give it a go as a child (even with no prior experience - swimming) and that has shaped me into the adult that I am which has big dreams and goes after them.

Caleb, where do I even begin? You are my best friend and I really wouldn't have made it through this without you in my life. We have endured 3000 km between us for the last four years, and yet our relationship and friendship continues to go from strength to strength. This journey has been hard and isolating, and you've been on the other end of the phone for all of it. I just can't thank you enough for continually encouraging me, sometimes every hour of every day, to just keep at it and get there. Thank you.

A core theme of this PhD candidature was creating a contraceptive which could improve animal welfare. I hope that my work can contribute to this field so that one day we no longer live in a society that condones irresponsible pet ownership or backyard breeding. That one day animal shelters aren't overrun, and animal cruelty is unheard of. That one day, the value of animal life is not measured by its dollar value. I look forward to that day.

~

I am a messenger for change; for our society to recognise the value of animal life

We have the scientific knowledge to create change

But only when we change within ourselves will we rid the world of animal cruelty

~

2013 - 2015

I dedicate this PhD thesis in the memory of Talulah, Clover, Delilah and Peppy.

Publications and awards arising from research in this thesis

1. Publications

CHAPTER 1

Hall SE, Nixon B, Aitken RJ (2016) Non-surgical sterilisation methods may offer a sustainable solution to feral horse *(Equus caballus)* overpopulation. *Reproduction, Fertility and Development* 10.1071/RD16200 **Published**

CHAPTER 2

Hall SE, Upton RO, McLaughlin EA, Sutherland JM (2017) PI3K/AKT and JAK/STAT follicular signalling is conserved in the mare ovary. *Reproduction, Fertility and Development* **Published**

CHAPTER 3

Hall SE, Gibb Z, Nixon B, Smith ND, Aitken RJ (2017) Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa. *Biology of Reproduction* 10.1095/biolreprod.116.145292 **Published**

CHAPTER 4

Hall SE, Gibb Z, Nixon B, Swegen A, Aitken RJ (2017) Active immunization with sperm proteins covalently modified by the lipid peroxidation product, acrolein.

2. Statements of contribution

By signing below I attest that the Higher Degree Research candidate Sally Hall has contributed upward of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

Laureate Professor R John Aitken

Date: 20/01/2017

Frances Martin, Assistant Dean Research Training

Date: 23/01/2017

Doctor Zamira Gibb

Date: 17/01/2017

Professor Brett Nixon

Date: 20/01/2017

Doctor Jessie Sutherland

Date: 17/01/2017

Professor Eileen McLaughlin

Date: 20/01/2017

Nathan Smith

Date: 17/01/2017

Aleona Swegen

Date: 17/01/2017

Rose Upton

Date: 16/01/2017

3. Conference proceedings relevant to this thesis

Hall SE, Gibb Z, Aitken RJ. Immunisation with aldehyde-adducted sperm proteins reduces sperm-egg binding in the mouse. 47th Annual Conference of the Society for Reproductive Biology, 21-24 August 2016, Gold Coast, Australia

Hall SE, Gibb Z, Nixon B, Aitken RJ. Electrophilic aldehydes increase free radical production and modify proteins in stallion spermatozoa. 18th International Congress of Animal Reproduction, 26-30 June 2016, Tours, France

Hall SE, Gibb Z, Nixon B, Aitken RJ. Electrophilic aldehydes increase free radical production and modify surface proteins in horse spermatozoa. 46th Annual Conference of the Society for Reproductive Biology, 23-26 August 2015, Adelaide, Australia

Hall SE, Gibb Z, Nixon B, Aitken RJ. Using aldehyde exposed sperm proteins as a permanent contraceptive for feral horse management. Invasive Animals Cooperative Research Centre Review, 23-25 March 2015, Canberra, Australia

Hall SE, Bernstein IR, Nixon B, McLaughlin EA, Aitken RJ. Improving livestock productivity and reducing invasive animal burden using phage peptides fertility control. 45th Annual Conference of the Society for Reproductive Biology, 24-27 August 2014, Melbourne, Australia

Hall SE, Bernstein IR, Aitken RJ, McLaughlin EA. Phage peptides fertility control: non-surgical sterilisation of female horses. Australasian Vertebrate Pest Conference, 26-29 May 2014, Brisbane, Australia

Hall SE, Sutherland JM, Aitken RJ, McLaughlin EA. Phage peptides fertility control: Non-surgical sterilisation of feral horses. 18th Annual RHD Conference, 6 December, 2013, Newcastle, Australia

Hall SE, Sutherland JM, Aitken RJ, McLaughlin EA. JAK/STAT signalling and suppressors of cytokine signalling in equine folliculogenesis. 44th Annual Conference of the Society for Reproductive Biology, 25-28 August 2013, Sydney, Australia

4. Additional co-authored publications

Hall SE, Negus C, Johinke D, Bathgate R (2017) Adjusting cryodiluent composition for improved post-thaw quality of rabbit spermatozoa. *PLoS ONE*

Gibb Z, Lambourne SR, Curry BJ, **Hall SE**, Aitken RJ (2016) Aldehyde Dehydrogenase Plays a Pivotal Role in the Maintenance of Stallion Sperm Motility. *Biology of Reproduction* 94(6):133, 1–11.

5. Awards

2016

Finalist in the Society for Reproductive Biology Oozoa award for best student presentation

Joint recipient of the People's Choice Award in the HMRI Thru the Lens science photography competition

NSW Semi-finalist for the FameLab science communication

2015

Finalist in the Society for Reproductive Biology Oozoa award for best student presentation

Finalist in the Society for Reproductive Biology Science Meets Publican competition

Invited speaker at the AusBiotech NSW BioBriefing

Finalist in the University of Newcastle Three Minute Thesis

2013

Third place in the University of Newcastle Three Minute Thesis

Invasive Animals Cooperative Research Centre PhD scholarship recipient

Table of Contents

Abstract

Feral horses currently require immediate population control in certain areas of Australia, and are a pest species across many regions of the world. Fertility control is considered the most suitable management strategy for control; however, agents currently available are largely non-sustainable for free-ranging feral horses. This is due to the difficulties associated with application of a fertility control agent, the ability to then identify animals which have been treated, and finally, the timely readministration of boosters. Consequently, there is an urgent need to develop a fertility control agent for feral horses that is easy to administer and will persist for the lifetime of the animal. While extensive research in the development of a non-surgical sterilisation method has been conducted in mouse models, little has been done in horses, creating a need for research to be undertaken investigating horse gamete biology. In this thesis, a fertility control agent for feral horse management was investigated from both a female and male perspective.

Initial recruitment at the beginning of folliculogenesis, responsible for the activation of primordial follicles within the ovary, is considered the fundamental phase that determines the reproductive potential of a female. There is an abundance of evidence to indicate that if recruitment from the primordial follicle population is suppressed or if the primordial follicle population is depleted then a state of infertility is induced. Recent work has shown that interruption of normal follicular processes via irregularities in signalling pathways such as phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) can have irrevocable consequences, resulting in infertility. Consequently, investigation into mare ovarian anatomy and follicular signalling processes was the primary approach taken to determine whether manipulation of these pathways could be used in the future development of a non-surgical sterilisation method. Messenger RNA expression of

signalling molecules within two signalling pathways essential for initial recruitment in folliculogenesis, *PI3K/AKT* and *janus kinase/signal transducer and activator of transcription (JAK/STAT)*, significantly increased between gestation and adulthood, coincident with sexual maturity. Protein localisation was also largely conserved which suggests that signalling pathway inhibitors can indeed be used as a means of manipulating the rate of primordial follicle activation as an approach to fertility control in this species.

Next we explored the impact of exogenous electrophilic aldehydes on stallion spermatozoa, as interruption of the delicate protein profile that exists within the plasma membrane has been demonstrated to cause infertility, often due to defective sperm-egg recognition and fusion. A significant increase in mitochondrial and cytosolic reactive oxygen species (ROS), lipid peroxidation and a commensurate loss of motility resulted following exposure to acrolein (ACR) and 4-hydroxynonenal (4HNE); partly due to perturbation of mitochondrial aldehyde dehydrogenase (ALDH2), a detoxification mechanism which is responsible for counteracting acute ROS formation. We identified proteins vulnerable to adduction and covalent modification, including: heat shock protein 90 alpha (cytosolic) class A member 1 (HSP90AA1) and arylsulfatase A (ARSA) by ACR and 4HNE, respectively. Zona pellucida binding competence was also lost following exposure to low levels of ACR and 4HNE.

Exposure to electrophilic aldehydes such as ACR and 4HNE has been associated with the development of autoimmune disease. Consequently, following on from the findings in the previous study, we next performed a fertility trial within the mouse model, immunising mice with proteins that had previously been exposed to ACR and 4HNE. Females immunised with ACR-adducted sperm proteins and subsequently mated experienced a reduction in the number of pregnancies, while sperm from mice immunised with ACR-adducted sperm proteins had significantly

reduced zona pellucida binding competence *in vitro*. Blood serum from males revealed antibodies against proteins that were covalently modified by ACR, which have been implicated in gamete interaction, including: heat shock protein 60 (HSP60) and arylsulfatase A (ARSA), izumo sperm-egg fusion 1 (IZUMO1) and protein disulfide isomerase A6 (PDIA6).

Taken together, the studies within this thesis have significantly contributed to our understanding of mare and stallion gamete biology. We have identified a critical role for electrophilic aldehydes in the adduction and covalent modification of sperm proteins critical for sperm-egg recognition. The use of covalently modified sperm proteins in a species-specific vaccine for feral horses would provide a novel means of fertility control that may potentially offer long-term contraceptive protection.

CHAPTER 1 – LITERATURE REVEW

Non-surgical sterilisation methods may offer a sustainable solution to feral horse (*Equus caballus*) overpopulation

Published: Reproduction, Fertility and Development (2016) 10.1071/RD16200

Authors: Hall SE, Nixon B, Aitken RJ

Chapter 1: Overview

Feral horses are a significant pest species in Australia, and across many parts of the world. Many methods of control have been implemented to manage feral horse populations, including mustering, trapping, and aerial shooting; however, they are met with considerable criticism because Brumbies are an important part of Australian heritage and in many circles regarded as native, despite their introduction during European settlement. The most effective method of feral horse control, aerial shooting, has been banned in many regions of Australia where feral horses are most abundant. This has created an urgent need to develop a feral horse management plan in Australia that, in the first instance, is publicly accepted, and most importantly, is effective in managing feral horses.

Fertility control has been researched extensively for feral horse management; however, its use in Australian feral horse populations is largely ineffective because of readministration requirements. Delivery of a fertility agent to free-ranging horses is extremely difficult, and the need to subsequently readminister the agent makes this practice highly inefficient. One way to overcome the issues surrounding fertility control is to create a contraceptive agent that once administered is persistent for the lifetime of the animal. Additionally, a non-surgical sterilant that can be delivered passively by baiting would offer the most cost effective method and likely result in high rates of public acceptance.

The aim of this chapter was to review current methods of fertility control for feral horses and offer insight into alternative contraceptive strategies that may be implemented for the management of this species. This review describes three main concepts by which non-surgical sterilisation could be achieved: the first involves manipulation of local gonadal signalling in the mare, the second is founded on random phage-peptide technology, and the third targets key proteins found on

spermatozoa or the conceptus for covalent modification by electrophilic aldehydes with the goal of enhancing their immunogenicity. The studies described in subsequent results chapters were designed with the goal of exploring the utility of these novel approaches to fertility management. Specifically, studies reported in Chapter 2 aimed to investigate local ovarian signalling in the mare. Similarly, the experiments reported in Chapters 3 and 4 were undertaken to demonstrate the potential efficacy of the latter strategy of using aldehyde adducted sperm proteins to elicit a potent immune response. Notably, for logistical reasons the latter proof-of-concept studies (Chapter 4) were conducted in a mouse model rather than the target species of the horse.

CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD16200

Review

Non-surgical sterilisation methods may offer a sustainable solution to feral horse (*Equus caballus*) overpopulation

Sally Elizabeth Hall[A,B], *Brett Nixon*[A] *and R. John Aitken*[A,B]

[A]Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, Life Sciences Building, University of Newcastle, Callaghan, NSW 2308, Australia.
[B]Invasive Animals Cooperative Research Centre, Building 22, University of Canberra, University Drive South, Bruce, ACT 2617, Australia.
[C]Corresponding author. Email: sally.e.hall@uon.edu.au

Abstract. Feral horses are a significant pest species in many parts of the world, contributing to land erosion, weed dispersal and the loss of native flora and fauna. There is an urgent need to modify feral horse management strategies to achieve public acceptance and long-term population control. One way to achieve this is by using non-surgical methods of sterilisation, which are suitable in the context of this mobile and long-lived species. In this review we consider the benefits of implementing novel mechanisms designed to elicit a state of permanent sterility (including redox cycling to generate oxidative stress in the gonad, random peptide phage display to target non-renewable germ cells and the generation of autoantibodies against proteins essential for conception via covalent modification) compared with that of traditional immunocontraceptive approaches. The need for a better understanding of mare folliculogenesis and conception factors, including maternal recognition of pregnancy, is also reviewed because they hold considerable potential in providing a non-surgical mechanism for sterilisation. In conclusion, the authors contend that non-surgical measures that are single shot and irreversible may provide a sustainable and effective strategy for feral horse control.

Additional keywords: fertility, immunocontraception, oxidative stress, primordial germ cells, spermatozoa.

Received 13 May 2016, accepted 9 September 2016, published online 5 October 2016

Introduction

Feral horses (*Equus caballus*) are a significant pest species in many parts of the world, particularly in the US, regions of Europe, Australia and New Zealand (Long 2003). The large, hard-hooved herbivore is responsible for considerable environmental damage (Dyring 1990; Andreoni 1998; English 2000), the spread of disease (Dobbie *et al.* 1993; Campbell *et al.* 2002; Cheng and Currie 2005; Dawson *et al.* 2006) and an indirect decline in native flora and fauna populations (Dobbie *et al.* 1993; Symanski 1994; Levin *et al.* 2002; Beever and Brussard 2004; Zalba and Cozzani 2004; Beever and Herrick 2006). A lack of predators and favourable environmental conditions can allow feral horse numbers to rise by 20% per annum (Eberhardt *et al.* 1982; Dobbie *et al.* 1993; Dawson *et al.* 2006; National Research Council 2013). In Australia, the population of feral horses is in excess of 600 000 brumbies, notably the largest feral horse population in the world (Berman and Jarman 1987; Clement *et al.* 1990; Dawson *et al.* 2006; Nimmo and Miller 2007). Integrated pest management strategies for feral horses include trapping and mustering, where animals are euthanased and sold for meat or trained and used as stock horses (Rolls 1969; Warren *et al.* 1993; Symanski 1994; Csurhes *et al.* 2009; Garrott and Oli 2013). However, the most extensively practiced method for population control is culling in the form of aerial shooting (English 2000; Dawson *et al.* 2006; English 2006). Although the requirements to be a shooter are high, the method remains contentious (Symanski 1994; Chapple 2005; Bradshaw *et al.* 2007; Nimmo and Miller 2007). Aerial shooting of feral horses is largely unaccepted by the public because free-ranging horses are often considered a cultural icon and an important part of national heritage (Berman 1991; Bomford and O'Brien 1997; Chapple 2005; Dawson *et al.* 2006; Bradshaw *et al.* 2007; Nimmo and Miller 2007; Killian *et al.* 2008). Furthermore, most control methods are laborious, expensive and ineffective, markedly failing to reduce the population growth rate of this pest species (Dobbie *et al.* 1993; Symanski 1994; Dawson *et al.* 2006; Killian *et al.* 2008; National Research Council 2013). Legal restrictions have been used to ban aerial shooting (English 2000; Chapple 2005), which has created an urgent need to implement secondary control strategies that are publicly accepted and offer a sustainable solution to feral horse overpopulation.

Considerable progress has been made in the area of immunocontraceptive development (Fagerstone *et al.* 2010; Gray and

Table 1. Summary of contraceptive options currently available and emerging for feral horse management
For each contraceptive, the duration of effect, efficacy and the sex to which the method pertains are listed. GnRH, gonadotrophin-releasing hormone; ZP, zona pellucida

Target	Contraceptive	Stage of development	Sex	No. doses	Contraceptive coverage	Efficacy (%)	References
GnRH	Equity	Established	Male and female	2	1 year	100	Elhay *et al.* (2007), Janett *et al.* (2009)
	Improvac	Established	Female	2	1–2 years	100	Imboden *et al.* (2006), Botha *et al.* (2008)
	GonaCon	Established	Male and female	1	1–4 years	40–94	Killian *et al.* (2006), Killian *et al.* (2008), Miller *et al.* (2008), Gray *et al.* (2010)
ZP	SpayVac	Established	Female	1	≥3 years	83–100	Powell and Monfort (2001), Killian *et al.* (2006), Killian *et al.* (2008), Turner *et al.* (2008), Gray *et al.* (2010), Bechert *et al.* (2013), Mask *et al.* (2015)
Proteins found on primordial follicles, spermatogonial stem cells, Sertoli cells	Phage peptides	Emerging	Male and female	1	Lifelong (intended)	–	Eidne *et al.* (2000), Meloen *et al.* (2000), Aitken (2006), Samoylova *et al.* (2010, 2012, 2012, 2015)
Proteins found on spermatozoa, oocyte and blastocyst	Aldehyde-adducted proteins	Future	Male and female	1	Lifelong (intended)	–	Dixit *et al.* (1975), Bohring and Krause (2003), Frank *et al.* (2005), Klein *et al.* (2010), Wang *et al.* (2012)

Cameron 2010; Kirkpatrick *et al.* 2011; Massei and Cowan 2014), but a sustainable and effective method for free-ranging populations has yet to be implemented successfully (Bomford and O'Brien 1997; English 2000; Dawson *et al.* 2006; Hinds 2006; Killian *et al.* 2008; Powers *et al.* 2011; National Research Council 2013). This is due, at least in part, to the need for frequent readministration. One way to overcome this is by using non-surgical methods of sterilisation, which are suitable in the context of this mobile and long-lived species (Dell'Omo and Palmery 2002; National Research Council 2013; Amory *et al.* 2014; Aitken 2015; Naz and Saver 2016). The success of such a formulation is likely predicated on its ability to induce a state of permanent and irreversible sterility. It is highly desirable that the sterilant is cell and species specific and capable of affecting both sexes uniformly to cause permanent sterility after a single application. Herein we briefly discuss fertility control vaccines currently available and review novel approaches that remove the need for readministration (Table 1). Novel methods of non-surgical sterilisation may prove preferable in the context of integrated management approaches to feral horse populations.

Fertility control currently used for feral horse management

Compared with human and domestic animal contraception, the implementation of effective fertility control strategies for feral horses encompasses several unique challenges (Killian *et al.* 2008; Kirkpatrick *et al.* 2011). Issues surrounding specificity, reversibility, methods of delivery and variability in response have restricted the widespread adoption of fertility control strategies as part of integrated management approaches (Stout and Colenbrander 2004). Notwithstanding these limitations, at least two contraceptive methods based on targeting critical aspects of gonadal regulation or gamete interaction have shown promise for feral horse management (Fig. 1).

The first gonadotrophin-releasing hormone (GnRH) immunisation in horses was conducted by Schanbacher and Pratt (1985) to assess its effect on cryptorchidism in stallions. The ensuing three decades saw a proliferation of GnRH-targeting strategies implemented for fertility control (Stout and Colenbrander 2004; Fagerstone *et al.* 2010), but many methods were largely discounted for free-ranging species in favour of vaccination regimens incorporating formulations of the GnRH peptide (or a conjugated form of the hormone; Miller *et al.* 2008; Powers *et al.* 2011, 2014). Pertinent for both sexes, GnRH formulations assessed in stallions have been shown to affect sperm motility acutely before resulting in a complete loss of libido, disrupted spermatogenesis and reduction in testis size, measured 2 weeks after the second immunisation (Turkstra *et al.* 2005).

Several commercial GnRH vaccines are currently in production, including Improvac (CSL), Equity (Pfizer Animal Health) and GonaCon (United States Department of Agriculture National Wildlife Research Centre). In studies by Imboden *et al.* (2006), two doses of Improvac in the mare permitted a contraceptive coverage of 23 weeks, but this coincided with an undesirable reaction to the adjuvant formulated into this commercial vaccine. Subsequent studies using Improvac reported no adverse reaction (Botha *et al.* 2008). Equity, a vaccine created specifically for horse contraception, generated better coverage (at least 1 year) with no adverse reactions to the immunisation (Elhay *et al.* 2007). Finally, 4 years after GonaCon delivery, 40% of mares experienced a continued contraceptive effect (Killian *et al.* 2008).

In the US, these vaccines are not commercially available, leading Donovan *et al.* (2013) to instead test a commercially available canine GnRH in mares. The findings of that study revealed the vaccine inhibited ovarian function, but also altered reproductive behaviours that are integral to the maintenance of the complex social structure of herd animals such as horses.

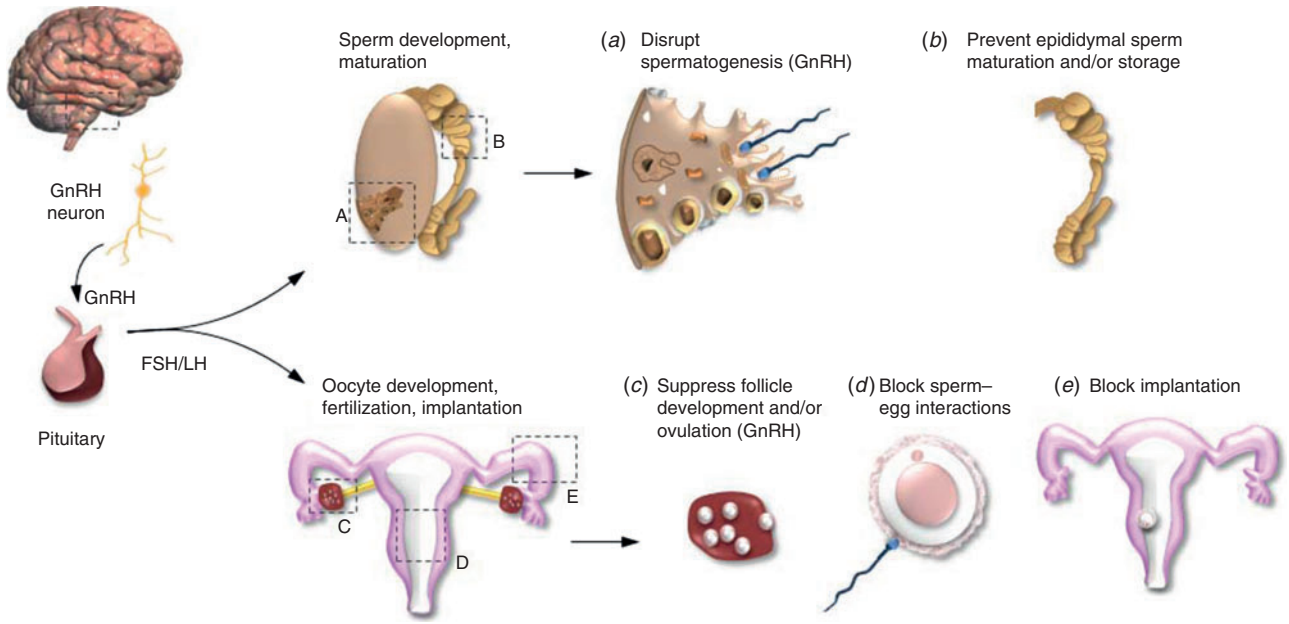


Fig. 1. Target sites for contraceptive intervention. In the male reproductive system, fertility control methods are used to disrupt spermatogenesis (gonadotrophin-releasing hormone (GnRH); A) or prevent epididymal sperm maturation and/or storage (B). In the female reproductive system, fertility control methods suppress follicle development and/or ovulation (C), block sperm–egg interactions (D) or block implantation (E).

Kisspeptin has also been investigated because it plays an important role in initiating GnRH secretion, but research is currently in its infancy and it is difficult to investigate in the horse (Decourt *et al.* 2008). Altering normal GnRH activity through the use of agonists has also proven quite difficult in mares (Fitzgerald *et al.* 1993) due to internalisation of GnRH and downregulation of GnRH receptors (Kutzler and Wood 2006; Finch *et al.* 2009). Additional issues are that these vaccines are neither tissue nor species specific (Kirkpatrick *et al.* 2011; Samoylova *et al.* 2012), and booster immunisations are required to maintain effective antibody titres (Herbert and Trigg 2005). A more promising approach has centred on the development of anti-zona pellucida (ZP) vaccines incorporating native formulations of whole ZP that have been harvested from the pig, a tissue resource that is readily available from abattoirs (Kutzler and Wood 2006; McLaughlin and Aitken 2011). Such vaccines exploit the high degree of species conservation among the ZP proteins and have been used on over 80 species, with variable degrees of efficacy (Kirkpatrick *et al.* 2011). This may be due to the unique differences in the number and localisation of ZP glycoproteins between species (Mugnier *et al.* 2009). The anti-ZP antibodies generated following immunisation bind antigenic sites on the outer surface of the ZP and thereby competitively inhibit sperm binding or prevent sperm accessing target ligands through a process of steric hindrance (Aitken and Richardson 1981; Liu *et al.* 1989).

Liu *et al.* (1989) first demonstrated the potential applicability of anti-porcine (p) ZP as an immunocontraceptive in captive mares following four injections of the vaccine. Subsequently, its efficacy in controlling feral horse populations was assessed in field trials targeting a herd of free-ranging horses inhabiting Assateague Island in the US (Kirkpatrick *et al.* 1990). That study demonstrated that remote delivery of the anti-pZP vaccine could successfully impede fertility of both pregnant and non-pregnant mares without affecting foetal health. These novel findings stimulated a series of elegant secondary studies assessing reversibility (Kirkpatrick and Turner 2002; Feh 2012), time to population decline (Kirkpatrick and Turner 2008), duration of contraceptive effect (Turner *et al.* 2008; Ransom *et al.* 2011) and changes to social behaviours (Nuñez *et al.* 2009, 2010; Madosky *et al.* 2010; Ransom *et al.* 2010). Contraceptive studies were conducted predominantly using a pelleted pZP vaccine called PZP-22, before registration of ZonaStat-H by the Humane Society of the United States as a contraceptive for free-ranging horses (Mask *et al.* 2015). However, the most recent commercially available pZP vaccine is SpayVac (IVT), a formulation that has demonstrated continued fertility control in feral horses for at least 3 years after a single administration (Killian *et al.* 2008; Bechert *et al.* 2013). Although directed against ZP glycoprotein 3 (ZP3), SpayVac is regarded as less pure than either PZP-22 or ZonaStat-H, potentially increasing its antigenicity and making it the best candidate for field application (National Research Council 2013; Mask *et al.* 2015).

In studies that have sought to directly compare the efficacy of anti-pZP vaccination with that of anti-GnRH for feral horse control, the former has generally proven more favourable (Bechert *et al.* 2013; National Research Council 2013). Two studies by Killian *et al.* (2006, 2008) found higher rates of infertility (83%) in mares 3 years after a single administration of SpayVac, compared with only 60% infertility after treatment with GonaCon. Notwithstanding these promising data, studies of anti-pZP have reported females exhibiting prolonged reproductive behaviours (Nuñez *et al.* 2010; Ransom *et al.* 2010), as well as decreased band fidelity, which significantly affects overall

harem stability (Nuñez *et al.* 2009; Madosky *et al.* 2010). Although such atypical behaviour may be a common feature of immunocontraceptive strategies (National Research Council 2013; Ransom *et al.* 2013), targeting the conserved molecular structure of the mammalian ZP proteins does impose additional limitations with regard to a lack of species specificity (Samoylova *et al.* 2012). Furthermore, the vaccine is only useful as a female contraceptive and the immunological response varies between individual animals treated (Kirkpatrick *et al.* 2012). Following SpayVac administration to eight mares, Mask *et al.* (2015) reported that although all produced elevated anti-ZP antibody titres, one mare was still able to conceive, inferring antibody titres are not a stand-alone representation of fertility cessation.

Additional consequences of anti-pZP immunisation tend to vary between species (Barber and Fayrer-Hosken 2000; Prasad *et al.* 2000), but include interference with follicle maturation and ovulation as well as primordial follicle depletion (Skinner *et al.* 1984; Paterson *et al.* 1998; Tung *et al.* 2002; Bechert *et al.* 2013). In feral horses, ovarian pathology was reported following 3 years of immunocontraception with anti-pZP (Powell and Monfort 2001). The mechanisms underpinning the induction of such pathologies warrant further investigation because they may provide insight into the development of a novel permanent fertility control agent that persists over the lifetime of the animal and thus removes the need for readministration.

Non-surgical sterilisation technologies

Induction of reproductive senescence

Most contraceptive strategies for animal population control require readministration (Asa *et al.* 2010) because they target regenerative proteins or secretory products, making the immune response a very inefficient process. Prolonging the efficacy of vaccines by lengthening the time to readministration or removing the need for readministration completely requires targeting non-renewable germ cells, and is a critically important consideration for long-term, sustainable feral horse control. Primordial follicles in the ovary and spermatogonial stem cells and Sertoli cells in the testis are essential for the expression of normal fertility. These cells represent ideal targets for fertility control in that they are terminally differentiated cell types that cannot be replenished by the reproductive system (Aitken and Curry 2011). Thus, once these cells have been depleted, the reproductive potential of the animal is lost (Meirow and Nugent 2001; Aitken *et al.* 2004; Sobinoff *et al.* 2010).

A variety of cellular insults have been identified that are capable of inducing germ cell depletion and hence infertility, including autoimmune disease, exposure to radiation, xenobiotics and other cytotoxic agents (Aitken *et al.* 2004; Silva *et al.* 2014). These insults generally lead to the production of free radicals, such as reactive oxygen species (ROS), and thus exploit the sensitivity of the developing gamete to a state of oxidative stress (Hoyer *et al.* 2001; Hanoux *et al.* 2007). The process of redox cycling has been well documented for a variety of xenobiotics, such as pesticides, insecticides, flame retardants, polycyclic aromatic hydrocarbons and dioxins (Hoyer *et al.* 2001; Aitken *et al.* 2004; Aitken 2008; Aitken and De Iuliis 2010). The generation of superoxide radicals begins a cascade of events leading to the formation of ROS, lipid peroxidation and subsequent electrophile generation (Gutierrez 2000; Aitken *et al.* 2012). Reagents responsible for redox cycling and the generation of oxidative stress could make ideal contraceptive agents (Aitken and Baker 2006), particularly in the case of horse spermatozoa, because they contain high levels of polyunsaturated fatty acids, rendering them highly susceptible to electrophile accumulation and DNA damage (Aitken *et al.* 2012).

Oxidative stress associated with excessive ROS generation and the induction of lipid peroxidation is an acknowledged cause of spontaneous infertility in human males (Aitken and Clarkson 1987; Moazamian *et al.* 2015). Similarly, in the female reproductive system, numerous studies have examined free radical production through redox cycling leading to oxidative stress capable of depleting the germ cell pool (Meirow and Nugent 2001; McLaughlin *et al.* 2003; Pujianto *et al.* 2010; Sobinoff *et al.* 2010; Kim *et al.* 2013). As a specific example, menadione, a redox active naphthoquinone, causes widespread oxidative stress in male and female gametes leading to significant DNA damage and loss of functionality (De Iuliis *et al.* 2006; Sobinoff *et al.* 2010; Aitken *et al.* 2013).

Such proof-of-concept studies provide evidence that a localised state of oxidative stress within the gonad can cause infertility. One technology that may prove amenable to selectively delivering this form of cellular insult to the gonad is that of random peptide phage display (Meloen *et al.* 2000). Initially introduced in a contraceptive context by Eidne *et al.* (2000), this technology was used to generate peptides against the sperm surface that bound to the equatorial segment and interfered with sperm–oocyte fusion. Subsequently, random peptide phage display technology was used in dogs (Samoylova *et al.* 2010) and pigs (Samoylova *et al.* 2012) and succeeded in identifying key sperm proteins that mediate adhesion to the ZP in these species. Similar techniques have also been used to increase the immunogenicity of GnRH (Sabeur *et al.* 2003), which has low antibody response due to its prevalence in the body (Samoylov *et al.* 2012). Although these reports highlight the usefulness of using targeting peptides to directly disrupt normal cellular signalling and affect fertility, there is also the opportunity to couple these peptides with redox cycling xenobiotics to ensure apoptosis of the target cells and non-surgical sterilisation (Aitken 2006). This strategy relies on iterative rounds of selection to identify bacteriophage expressing peptide inserts that bind to the target cell types (e.g. the pregranulosa cells and oocyte that make up primordial follicles in the ovary, and spermatogonial stem cells and Sertoli cells in the testis) with a high degree of affinity and specificity (Eidne *et al.* 2000; Samoylova *et al.* 2012). These peptides are then conjugated to xenobiotics engineered in a manner that permits their delivery only in the immediate vicinity of the target cell, thereby ensuring both the efficacy and safety of the approach (Humphrys and Lapidge 2008). Substantial progress with this peptide technology has been achieved in recent years, enabling xenobiotics to be coupled to phage peptides in such a way that they can be targeted directly to the gonad with a single intraperitoneal (i.p.) injection. For example, in studies by Amory *et al.* (2014), an FSHβ–melphalan peptide conjugate suppressed normal spermatogenesis in mice.

Another mechanism to recruit non-renewable germs cells is via local activation of signalling molecules within the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (Reddy *et al.* 2008; McLaughlin and McIver 2009; Sobinoff *et al.* 2012). During folliculogenesis, activation of the PI3K/AKT/mTOR pathway determines the rate of primordial follicle activation, mediated by phosphatase and tensin homologue (PTEN) phosphorylation (Keating *et al.* 2009; Sobinoff *et al.* 2010; Kim *et al.* 2013). Uncontrolled activation of the PI3K/AKT/mTOR pathway has been postulated as a main cause for premature ovarian failure. In a study by Adhikari *et al.* (2012), bisperoxovanadium, a known PTEN inhibitor, directly disrupted primordial follicle senescence by activating the PI3K/AKT/mTOR pathway. Exposure to 4-vinylcyclohexene diepoxide also delivered this effect, depleting ovarian follicle number in murine studies (Hoyer *et al.* 2001; Mayer *et al.* 2002; Dyer *et al.* 2013). Importantly, unregulated activation of this pathway has been associated with a concomitant increase in ROS production (Kim *et al.* 2013). Delivery of a local PTEN inhibitor could be achieved using peptides targets, but it would first be necessary to ensure gonadal signalling processes that are well characterised in the mouse model are conserved and function in the same manner in the horse. Indeed, little is known about horse gametogenesis compared with other species and, although many mechanisms are highly conserved, underpinning the mechanisms behind both spermato- and folliculo- genesis is crucial to the development of these technologies.

Generation of autoantibodies

A vaccine's efficacy is underpinned by its ability to present a foreign antigen to the body in such a way that it elicits an immune response that leaves the animal with lifelong immunity (Muller *et al.* 1997; Tung *et al.* 1999; Hardy *et al.* 2006). However, in the case of autoimmunity, events can occur whereby the immune system will fail to maintain tolerance and mount an immune response against self-antigens. Indeed, ovarian autoantibodies have been cited as responsible for reproductive failure in several cases, eliciting autoimmune oophoritis and premature ovarian failure (Garza and Tung 1995; Forges *et al.* 2004; Bakalov *et al.* 2005; Altuntas *et al.* 2006; Silva *et al.* 2014). Similarly, with regard to males, autoantibodies against sperm proteins are often generated in the male if the blood–testis barrier is breached (e.g. vasectomy; Bohring and Krause 2003). Spermatozoa are unique in that they are generated after immunological tolerance has been established in the body. Therefore, these cells have value as targets for fertility regulation because they are potentially antigenic (Alexander and Anderson 1979). In a study by Bohring and Krause (2003), the presence of anti-sperm antibodies was found to interfere with factors essential for fertilisation, including sperm morphology, sperm–oolemma binding ability and acrosome integrity. A variety of proteins have been investigated for their importance in reproductive processes and it is becoming increasingly evident that the development of these immune responses involves the targeting of a multitude of proteins, rather than singular entities, that are essential in sperm–egg binding and fertilisation (Nixon *et al.* 2007). Although oocyte- and ovarian-specific proteins are difficult to identify, many sperm-specific antigens have been discovered (Bohring and Krause 2003). An extensive overview of promising protein targets for immunocontraception in the horse can be found elsewhere (Swegen and Aitken 2016).

For feral horse control, the potential to identify antigens present on terminally differentiated cells allows for the development of innovative contraceptives with lifelong effectiveness. However, we are currently faced with a challenge of not only defining the precise subset of proteins involved in sperm–egg binding, but also delivering them to the immune system in such a manner that will elicit an immune response significant enough to cause infertility. Therefore, a method that can identify proteins essential for conception holds considerable promise. In a study by Wang *et al.* (2012), proteins were found to generate immune responses once they had been covalently modified as a result of becoming alkylated by electrophilic lipid aldehydes, such as 4-hydroxynonenal (4HNE) and malondialdehyde. In particular, Wang *et al.* (2012) found that aldehyde-modified proteins were capable of eliciting autoantibody formation and thus contribute to autoimmune disease pathogenesis. In this instance, autoimmunity arises because immunological tolerance to important protein antigens has been compromised (McLaughlin and Aitken 2011). By altering protein composition in such a way as to break tolerance, aldehyde-modified proteins are capable of eliciting an increase in immunogenicity that could result in a long-term contraceptive effect and even permanent sterility. This principle was established many years ago in dogs, where alkylation of sperm proteins using α-chlorohydrin caused lesions in the testis inhibiting spermatogenesis (Dixit *et al.* 1975). The concept of using aldehyde-modified sperm proteins as immunogens for long-term feral horse fertility control is promising given the evidence that pathological autoimmunity can be elicited in instances where proteins have become modified as a consequence of oxidative damage or adduction with small molecular mass metabolites, such as 4HNE or homocysteine thiolactone (Jakubowski 2010; Aitken *et al.* 2012; Gibb *et al.* 2014). We propose that aldehyde adducts on what are otherwise unknown proteins could be exploited to identify key proteins involved in conception and break immune tolerance to induce a state of autoimmunity in horses.

Maternal recognition of pregnancy

In the early stages of pregnancy, a process commonly referred to as 'maternal recognition of pregnancy' is initiated, whereby the conceptus secretes messages to promote the continued production of progesterone by the corpus luteum (CL), which is essential for supporting early embryonic development and survival (Bazer *et al.* 1994; Aitken 2002; McLaughlin *et al.* 2003; Gupta and Bansal 2010). In many animals, the maternal recognition of pregnancy is well understood. For example in ruminants, mononucleate cells of the trophectoderm release large amounts of interferon-τ (IFNT), which suppresses prostaglandin (PG) $F_{2\alpha}$ release from the endometrium (Roberts *et al.* 1996; Spencer and Bazer 2004). In comparison, the pig trophoblast produces oestrogen as it elongates in the uterine horn, which counteracts the effects of $PGF_{2\alpha}$ on the CL (Bazer *et al.* 1994; Klein *et al.* 2010; Swegen and Aitken 2016). However, the trophoblast of the horse

conceptus is unable to elongate due to internal turgor pressure (Allen and Wilsher 2009). Instead, $PGF_{2\alpha}$ and PGE_2 stimulate peristaltic contractions of the myometrium, allowing the embryo to travel around the uterus 12–14 times per day, sending signals to prevent luteolysis (Roberts *et al.* 1996; Stout and Allen 2001; Allen and Wilsher 2009; Klein *et al.* 2010). Many studies have tried to determine the nature of the factors emanating from the conceptus; however, the exact repertoire of signalling molecules released by the horse conceptus and their mechanisms of action remain to be fully resolved (Allen 1982; Bazer *et al.* 1994; Roberts *et al.* 1996; Allen and Wilsher 2009; Ealy *et al.* 2010; Klein *et al.* 2010; Swegen and Aitken 2016). A study by Ealy *et al.* (2010) provides strong evidence to suggest that the unknown signal, whatever it may be, likely targets the enzyme PGG/H synthase 2 to prevent luteolysis.

Using the technologies mentioned previously, it may be possible to increase the immunogenicity of as yet unknown proteins found on the blastocyst surface, perform immunisations and determine reproductive outcomes. This technology has been assessed previously, whereby peptides were developed to mimic trophoblast epitopes and their antigenicity tested by assessing their binding affinity to autoantibodies (Frank *et al.* 2005). Rather than interrupting conception, this approach inhibited the implantation of a successfully fertilised embryo. This later stage intervention alleviates any concern for changes to social dynamics and behaviours, but public acceptance of such an approach will need to be assessed to ensure its feasibility as a contraceptive for feral horses (Fagerstone *et al.* 2010). Using novel phage panning and aldehyde-modification technologies, we have the opportunity to identify proteins essential for the horse maternal recognition of pregnancy and exploit these to prevent embryo implantation, if and when fertilisation occurs.

Non-surgical sterilisation implementation and feasibility

Research into the molecular mechanisms governing mammalian reproduction has provided unprecedented insight into how reproductive processes may be compromised for the purpose of achieving long-term fertility control in feral horses. Indeed, the use of non-surgical sterilisation offers significant advantages in that it is cheaper to use than alternative methods, respects animal welfare and can be more effective than culling (Hobbs *et al.* 2000; Turner *et al.* 2001). However, incorporating non-surgical methods into our existing armoury of integrated pest management strategies faces several challenges, including stringent assessment of feral horse numbers and contraceptive suitability based on changes to reproductive cycling, animal behaviour and disruption to social dynamics between horses (Kirkpatrick and Turner 2008; Zabek *et al.* 2014*a*). As highly social animals, it remains imperative that we understand how management strategies including non-surgical sterilisation techniques may alter feral horse social structure (Madosky *et al.* 2010). It is difficult to ascertain whether compensatory factors, such as increased survival, may occur after the burden of fertility is removed from target populations (Warren *et al.* 1993; Ransom *et al.* 2014), as it has done following previous immunocontraceptive delivery (Turner and Kirkpatrick 2002; Kirkpatrick and Turner 2007), or whether selective pressure will manipulate the long-term effect of a particular contraceptive agent (Turner and Kirkpatrick 2002; Kirkpatrick and Turner 2008). However, this has not been the case in previous studies (Ransom *et al.* 2014) and changes to population demographics and social hierarchy issues have been reviewed extensively (Berman 1991; Dobbie *et al.* 1993; Fagerstone 2002; Asa and Porton 2005; Kirkpatrick *et al.* 2011; Massei and Cowan 2014).

Perhaps one of the greatest obstacles to the implementation of an efficacious contraceptive strategy is the development of a suitable delivery method that is both practical and cost-effective (Bomford and O'Brien 1997; Lapidge *et al.* 2010). Remote delivery has been thoroughly investigated for its applicability, regarded as an ideal method for reducing labour costs and minimising stress experienced by animals (Kirkpatrick *et al.* 1990, 1996, 2011). However, an in-depth review by the National Research Council (2013) describes the shortcomings of this technique, concluding that it is unlikely to be suitable for feral horses owing to the complexities associated with successful administration (Garrott 1995; Nimmo *et al.* 2007). Indeed, remote delivery also poses an environmental contamination concern, because most darts fall out of the animal and are unrecovered (Warren *et al.* 1993). Oral baiting may ultimately be the only effective delivery mechanism for free-ranging horse populations (Killian *et al.* 2006; Humphrys and Lapidge 2008; Samoylova *et al.* 2012), but it is seldom discussed for incorporation into feral horse management strategies. Peptide technology certainly meets all the requirements for oral delivery under field conditions in that it is species specific, has shown oral activity and is environmentally safe (Humphrys and Lapidge 2008). Although oral baiting mitigates the problems that apply to remote delivery, it is not without its own limitations. For example, previous work has shown that baiting is complicated by the behavioural characteristics of feral horses, which exhibit a natural tendency to avoid unknown roughage, such as hay, even when presented to them in periods of drought (Hampson *et al.* 2011). In certain instances, bait delivery may alter normal behaviour, where animals associate bait with helicopters and avoid that particular form of roughage in the future (Kirkpatrick 2007). It is therefore important to integrate suitable bait systems that are enticing to horses and are likely to be actively consumed. An important consideration for the manufacturer of such a baiting system is that both the active agent and the oral bait itself are long lasting and capable of resisting exposure to fluctuations in temperature and humidity (Humphrys and Lapidge 2008). In this regard, it is anticipated that a large block would be more appropriate than pellets, because a block would expose less surface area to the environment and be more resistant to desiccation. In pilot studies in Australia, this bait structure has been successfully implemented as part of a pest management strategy for feral deer and goats (Hunt *et al.* 2014). Although results are promising, they dictate the contraceptive agent used in the bait will have to elicit a species specific, or at least species restricted, response to avoid unintentional effects on non-target domestic and native species. Consideration of the use of adjuvants will also need to be addressed, as will the possibility of being able to deliver a contraceptive that does not contain an adjuvant and thus eliminate adverse reactions (Kirkpatrick 2007). One further consideration is that the contraceptive agent is entirely consumed within the

target species and not prone to bioaccumulation, should the animal be collected at a later point and meat sold for pet consumption (Warren *et al.* 1993; Garrott 1995).

A non-surgical sterilisation method allowing lifelong coverage for feral horses would be incredibly valuable to industry stakeholders, but it must be ensured that the contraceptive agent is not passed from the animal in waste and does not taint agricultural land and water systems. The feasibility of any new management plan is also predicated on in-depth field studies into population demographics and distribution. There is still much to learn about feral horse ecology, which has been impeded by the remote habitats in which the feral horses typically reside (Zabek *et al.* 2014*b*). Traditional immunocontraceptive strategies have primarily focused on reducing female fertility, which is appropriate in the context of feral horse herd structure. However, the opportunity to impede the fertility of both sexes simultaneously is a feature that has not been offered previously, and poses the greatest opportunity for successful control (Dell'Omo and Palmery 2002). Such studies will likely be complemented by simulated modelling approaches to inform the level of contraceptive efficacy needed to achieve the desired reduction in horse numbers and the most suitable timing for introduction of the contraceptive into the population (Garrott 1995; Hone 1999; Hobbs *et al.* 2000; Ballou *et al.* 2008). Early work by Bomford and O'Brien (1997) highlighted the merits of implementing a non-surgical sterilisation method following periods of severe drought when feral horse numbers have declined and when the density of animals is likely to increase around watering holes. This was also suggested by Berman (1991) and the National Research Council (2013). Integrated feral horse management beginning with a population decline followed by intensive delivery of a non-surgical sterilant to both sexes is likely to prove the most successful in achieving milestones for feral horse control.

Contraceptive approaches are likely to continue to need public and political approval (Powers *et al.* 2014), a major obstacle that limits the application of current methods that rely on culling via aerial shooting (Nimmo and Miller 2007). It is likely that at the time of administration animals will be pregnant. Therefore, it is important to ensure contraceptives administered mid-gestation will cover females against future pregnancy, but not affect the health of the current foetus (Powers *et al.* 2011).

Feral horse population control is a long-term project, where integrated pest management strategies incorporating non-surgical sterilisation methods will require adequate funding and resource investment before a significant population reduction is seen. Previous contraceptive application in other species has been very costly, so it is important to develop new, cost-effective and sustainable technologies for implementation in population management strategies. Feral horse management is not a short-term issue and thus long-term solutions, such as non-surgical sterilisation, will likely have important roles in containing future population growth.

Conclusion

Current control methods for feral horses meet with considerable public criticism, which has provided the impetus to explore novel contraceptive strategies. Non-surgical methods of sterilisation offer an alternative and sustainable approach based on endocrine disruption of gamete development or immunological targeting of gamete interaction. Herein we provide a review of novel mechanisms designed to elicit a state of permanent sterility, including redox cycling to generate oxidative stress in the gonad, random peptide phage display to target non-renewable germ cells and generation of autoantibodies against proteins essential for conception via modification following aldehyde alkylation. Such approaches have shown considerable promise in a laboratory setting, but successful application in free-ranging, long-lived species such as the feral horse presents several unique obstacles. No single management strategy will be a panacea for feral horse control; management strategies will need to be revised frequently and incorporated together to achieve this long-term goal.

References

Adhikari, D., Gorre, N., Risal, S., Zhao, Z., Zhang, H., Shen, Y., and Liu, K. (2012). The safe use of a PTEN inhibitor for the activation of dormant mouse primordial follicles and generation of fertilizable eggs. *PLoS One* **7**(6), e39034. doi:10.1371/JOURNAL.PONE.0039034

Aitken, R. J. (2002). Immunocontraceptive vaccines for human use. *J. Reprod. Immunol.* **57**, 273–287. doi:10.1016/S0165-0378(02)00010-4

Aitken, R. J., inventor. (19 June 2006). University of Newcastle, assignee. Method for reducing the reproductive potential of a female animal. Australian patent application no. 2006903307.

Aitken, R. J. (2008). Just how safe is assisted reproductive technology for treating male factor infertility? *Expert Rev. Obstet. Gynecol.* **3**, 267–271. doi:10.1586/17474108.3.3.267

Aitken, R. J. (2015). Reagents for the sterilization of pest animal species. In 'Research Portfolio Summary 2014–15'. (Ed. Invasive Animals Cooperative Research Centre.) pp. 46–47. (Invasive Animals Cooperative Research Centre: Canberra.)

Aitken, R. J., and Baker, M. A. (2006). Oxidative stress, sperm survival and fertility control. *Mol. Cell. Endocrinol.* **250**, 66–69. doi:10.1016/J.MCE.2005.12.026

Aitken, R. J., and Clarkson, J. S. (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.* **81**, 459–469. doi:10.1530/JRF.0.0810459

Aitken, R. J., and Curry, B. J. (2011). Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid. Redox Signal.* **14**, 367–381. doi:10.1089/ARS.2010.3186

Aitken, R. J., and De Iuliis, G. N. (2010). On the possible origins of DNA damage in human spermatozoa. *Mol. Hum. Reprod.* **16**, 3–13. doi:10.1093/MOLEHR/GAP059

Aitken, R. J., and Richardson, D. W. (1981). Mechanism of sperm-binding inhibition by anti-zona antisera. *Gamete Res.* **4**, 41–47. doi:10.1002/MRD.1120040107

Aitken, R. J., Koopman, P., and Lewis, S. E. (2004). Seeds of concern. *Nature* **432**, 48–52. doi:10.1038/432048A

Aitken, R. J., Gibb, Z., Mitchell, L. A., Lambourne, S. R., Connaughton, H. S., and De Iuliis, G. N. (2012). Sperm motility is lost *in vitro* as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol. Reprod.* **87**, 110. doi:10.1095/BIOLREPROD.112.102020

Aitken, R. J., Smith, T. B., Lord, T., Kuczera, L., Koppers, A. J., Naumovski, N., Connaughton, H., Baker, M. A., and Iuliis, G. N. (2013). On methods for the detection of reactive oxygen species

generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology* **1**, 192–205. doi:10.1111/J.2047-2927.2012.00056.X

Alexander, N. J., and Anderson, D. J. (1979). Vasectomy: consequences of autoimmunity to sperm antigens. *Fertil. Steril.* **32**, 253–260. doi:10.1016/S0015-0282(16)44228-7

Allen, W. R. (1982). Immunological aspects of the endometrial cup reaction and the effect of xenogeneic pregnancy in horses and donkeys. *J. Reprod. Fertil. Suppl.* **31**, 57–94.

Allen, W. R., and Wilsher, S. (2009). A review of implantation and early placentation in the mare. *Placenta* **30**, 1005–1015. doi:10.1016/J.PLACENTA.2009.09.007

Altuntas, C. Z., Johnson, J. M., and Tuohy, V. K. (2006). Autoimmune targeted disruption of the pituitary–ovarian axis causes premature ovarian failure. *J. Immunol.* **177**, 1988–1996. doi:10.4049/JIMMUNOL.177.3.1988

Amory, J. K., Hong, S., Yu, X., Muller, C. H., Faustman, E., and Goldstein, A. (2014). Melphalan, alone or conjugated to an FSH-β peptide, kills murine testicular cells *in vitro* and transiently suppresses murine spermatogenesis *in vivo*. *Theriogenology* **82**, 152–159. doi:10.1016/J.THERIOGENOLOGY.2014.03.014

Andreoni, F. (1998). 'Evaluating Environmental Consequences of Feral Horses in Guy Fawkes River National Park.' (Department of Ecosystem Management, University of New England: Armidale, NSW.)

Asa, C. S., and Porton, I. J. (2005). 'Wildlife Contraception: Issues, Methods, and Applications.' (JHU Press.)

Asa, C. S., Porton, I. J., Baker, A., and Plotka, E. (2010). Contraception as a management tool for controlling surplus animals. In 'Wild Mammals in Captivity: Principles and Techniques for Zoo Management'. 2nd edn. (Eds D. Kleiman, K. Thompson and C. Kirk Baer.) pp. 469–482. (The University of Chicago Press: Chicago and London.)

Bakalov, V. K., Anasti, J. N., Calis, K. A., Vanderhoof, V. H., Premkumar, A., Chen, S., Furmaniak, J., Smith, B. R., Merino, M. J., and Nelson, L. M. (2005). Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. *Fertil. Steril.* **84**, 958–965. doi:10.1016/J.FERTNSTERT.2005.04.060

Ballou, J. D., Traylor-Holzer, K., Turner, A., Malo, A. F., Powell, D., Maldonado, J., and Eggert, L. (2008). Simulation model for contraceptive management of the Assateague Island feral horse population using individual-based data. *Wildl. Res.* **35**, 502–512. doi:10.1071/WR07124

Barber, M. R., and Fayrer-Hosken, R. A. (2000). Possible mechanisms of mammalian immunocontraception. *J. Reprod. Immunol.* **46**, 103–124. doi:10.1016/S0165-0378(99)00063-7

Bazer, F. W., Ott, T. L., and Spencer, T. E. (1994). Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. *Theriogenology* **41**, 79–94. doi:10.1016/S0093-691X(05)80052-4

Bechert, U., Bartell, J., Kutzler, M., Menino, A., Bildfell, R., Anderson, M., and Fraker, M. (2013). Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses. *J. Wildl. Manage.* **77**, 1386–1400. doi:10.1002/JWMG.600

Beever, E. A., and Brussard, P. F. (2004). Community- and landscape-level responses of reptiles and small mammals to feral-horse grazing in the Great Basin. *J. Arid Environ.* **59**, 271–297. doi:10.1016/J.JARIDENV.2003.12.008

Beever, E., and Herrick, J. (2006). Effects of feral horses in Great Basin landscapes on soils and ants: direct and indirect mechanisms. *J. Arid Environ.* **66**, 96–112. doi:10.1016/J.JARIDENV.2005.11.006

Berman, D. (1991) 'The Ecology of Feral Horses in Central Australia.' (University of New England: Armidale, NSW.)

Berman, D. M., and Jarman, P. (1987). Feral horses in the Northern Territory, Vol. 1. Ecology of feral horses in Central Australia and their interaction with cattle. University of New England, Report to Conservation Commission of the Northern Territory, Alice Springs.

Bohring, C., and Krause, W. (2003). Immune infertility: towards a better understanding of sperm (auto)-immunity The value of proteomic analysis. *Hum. Reprod.* **18**, 915–924. doi:10.1093/HUMREP/DEG207

Bomford, M., and O'Brien, P. (1997). Potential use of contraception for managing wildlife pests in Australia. In 'Contraception in Wildlife Management'. (Ed. J. S. Wintermute) pp. 205–214. (US Department of Agriculture)

Botha, A. E., Schulman, M. L., Bertschinger, H. J., Guthrie, A. J., Annandale, C. H., and Hughes, S. B. (2008). The use of a GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions. *Wildl. Res.* **35**, 548–554. doi:10.1071/WR07136

Bradshaw, C. J., Field, I. C., Bowman, D. M., Haynes, C., and Brook, B. W. (2007). Current and future threats from non-indigenous animal species in northern Australia: a spotlight on World Heritage Area Kakadu National Park. *Wildl. Res.* **34**, 419–436. doi:10.1071/WR06056

Campbell, G. L., Marfin, A. A., Lanciotti, R. S., and Gubler, D. J. (2002). West Nile virus. *Lancet Infect. Dis.* **2**, 519–529. doi:10.1016/S1473-3099(02)00368-7

Chapple, R. (2005). The politics of feral horse management in Guy Fawkes River National Park, NSW. *Aust. Zool.* **33**, 233–246. doi:10.7882/AZ.2005.020

Cheng, A. C., and Currie, B. J. (2005). Melioidosis: epidemiology, pathophysiology, and management. *Clin. Microbiol. Rev.* **18**, 383–416. doi:10.1128/CMR.18.2.383-416.2005

Clement, R. F., Doyle, K. A., and Murray, J. G. (1990). The significance of a major outbreak of quarantinable disease to the Australian horse industries. *Aust. Vet. J.* **67**, 77–78. doi:10.1111/J.1751-0813.1990.TB07707.X

Csurhes, S., Paroz, G., and Markula, A. (2009). 'Pest Animal Risk Assessment: Feral Horse.' (Queensland Government.)

Dawson, M., Lane, C., and Saunders, G. (2006). XXX. In 'Proceedings of the National Feral Horse Management Workshop', August 2006, Canberra, ACT. (Eds M. Dawson, C. Lane and G. Saunders) pp. 1–82. (Invasive Animals Cooperative Research Centre.)

De Iuliis, G. N., Wingate, J. K., Koppers, A. J., McLaughlin, E. A., and Aitken, R. J. (2006). Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. *J. Clin. Endocrinol. Metab.* **91**, 1968–1975. doi:10.1210/JC.2005-2711

Decourt, C., Tillet, Y., Caraty, A., Franceschini, I., and Briant, C. (2008). Kisspeptin immunoreactive neurons in the equine hypothalamus: interactions with GnRH neuronal system. *J. Chem. Neuroanat.* **36**, 131–137. doi:10.1016/J.JCHEMNEU.2008.07.008

Dell'Omo, G., and Palmery, M. (2002). Fertility control in vertebrate pest species. *Contraception* **65**, 273–275. doi:10.1016/S0010-7824(02)00285-8

Dixit, V. P., Lohiya, N. K., Arya, M., and Agrawal, M. (1975). Reversible changes in the testes and epididymides of dog treated with alpha-chlorohydrin. *Acta Biol. Med. Ger.* **34**, 1851–1856.

Dobbie, W. R., Berman, D. M., and Braysher, M. L. (1993). 'Managing Vertebrate Pests: Feral Horses.' (Australian Government Publishing Services: Canberra, ACT.)

Donovan, C. E., Hazzard, T., Schmidt, A., LeMieux, J., Hathaway, F., and Kutzler, M. A. (2013). Effects of a commercial canine gonadotropin releasing hormone vaccine on estrus suppression and estrous behavior in mares. *Anim. Reprod. Sci.* **142**, 42–47. doi:10.1016/J.ANIREPROSCI.2013.09.002

Dyer, C. A., Raymond-Whish, S., Schmuki, S., Fisher, T., Pyzyna, B., Bennett, A., and Mayer, L. P. (2013). Accelerated follicle depletion *in vitro* and *in vivo* in Sprague-Dawley rats using the combination of 4-vinylcyclohexene diepoxide and triptolide. *J. Zoo Wildl. Med.* **44**(Suppl.), S9–S17. doi:10.1638/1042-7260-44.4S.S9

Dyring, J. (1990). The impact of feral horses (*Equus caballus*) on sub-alpine and montane environments in Australia. PhD Thesis, University of Canberra.

Ealy, A. D., Eroh, M. L., and Sharp, D. C., III. (2010). Prostaglandin H synthase Type 2 is differentially expressed in endometrium based on pregnancy status in pony mares and responds to oxytocin and conceptus secretions in explant culture. *Anim. Reprod. Sci.* **117**, 99–105. doi:10.1016/J.ANIREPROSCI.2009.03.014

Eberhardt, L., Majorowicz, A., and Wilcox, J. (1982). Apparent rates of increase for two feral horse herds. *J. Wildl. Manage.* **46**, 367–374. doi:10.2307/3808648

Eidne, K. A., Henery, C. C., and Aitken, R. J. (2000). Selection of peptides targeting the human sperm surface using random peptide phage display identify ligands homologous to ZP3. *Biol. Reprod.* **63**, 1396–1402. doi:10.1095/BIOLREPROD63.5.1396

Elhay, M., Newbold, A., Britton, A., Turley, P., Dowsett, K., and Walker, J. (2007). Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH. *Aust. Vet. J.* **85**, 39–45. doi:10.1111/J.1751-0813.2006.00092.X

English, A. (2000). Report on the cull of feral horses in Guy Fawkes River National Park in October 2000. University of Sydney, Sydney.

English, A. W. (2006). A report on the management of feral horses in National Parks in New South Wales. NSW National Parks & Wildlife Service, Sydney.

Fagerstone, K. (2002). Wildlife fertility control. USDA National Wildlife Research Center, Staff Publications, Paper 489. Available at http://digitalcommons.unl.edu/icwdm_usdanwrc/489/ [verified 19 September 2016].

Fagerstone, K. A., Miller, L. A., Killian, G., and Yoder, C. A. (2010). Review of issues concerning the use of reproductive inhibitors, with particular emphasis on resolving human-wildlife conflicts in North America. *Integr. Zool.* **5**, 15–30. doi:10.1111/J.1749-4877.2010.00185.X

Feh, C. (2012). Delayed reversibility of PZP (porcine zona pellucida) in free-ranging Przewalski's horse mares. In 'International Wild Equid Conference. Vienna, Austria: University of Veterinary Medicine', 18–22 September 2012. p. 69. Available at https://www.vetmeduni.ac.at/fileadmin/v/fiwi/Konferenzen/Wild_Equid_Conference/IWEC_book_of_abstracts_final.pdf [verified 21 September 2016]

Finch, A. R., Caunt, C. J., Armstrong, S. P., and McArdle, C. A. (2009). Agonist-induced internalization and downregulation of gonadotropin-releasing hormone receptors. *Am. J. Physiol. Cell Physiol.* **297**, C591–C600. doi:10.1152/AJPCELL.00166.2009

Fitzgerald, B. P., Peterson, K. D., and Silvia, P. J. (1993). Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: preliminary evidence on suppression of ovulation during the breeding season. *Am. J. Vet. Res.* **54**, 1746–1751.

Forges, T., Monnier-Barbarino, P., Faure, G., and Bene, M. (2004). Auto-immunity and antigenic targets in ovarian pathology. *Hum. Reprod. Update* **10**, 163–175. doi:10.1093/HUMUPD/DMH014

Frank, H.-G., Bose, P., Albieri-Borges, A., Borges, M., Greindl, A., Neulen, J., Pötgens, A. J., and Kaufmann, P. (2005). Evaluation of fusogenic trophoblast surface epitopes as targets for immune contraception. *Contraception* **71**, 282–293. doi:10.1016/J.CONTRACEPTION.2004.12.022

Garrott, R. A. (1995). Effective management of free-ranging ungulate populations using contraception. *Wildl. Soc. Bull.* **23**, 445–452.

Garrott, R. A., and Oli, M. K. (2013). A critical crossroad for BLM's wild horse program. *Science* **341**, 847–848. doi:10.1126/SCIENCE.1240280

Garza, K. M., and Tung, K. (1995). Frequency of molecular mimicry among T cell peptides as the basis for autoimmune disease and autoantibody induction. *J. Immunol.* **155**, 5444–5448.

Gibb, Z., Lambourne, S. R., and Aitken, R. J. (2014). The paradoxical relationship between stallion fertility and oxidative stress. *Biol. Reprod.* **91**, 77. doi:10.1095/BIOLREPROD.114.118539

Gray, M. E., and Cameron, E. Z. (2010). Does contraceptive treatment in wildlife result in side effects? A review of quantitative and anecdotal evidence. *Reproduction* **139**, 45–55. doi:10.1530/REP-08-0456

Gray, M. E., Thain, D. S., Cameron, E. Z., and Miller, L. A. (2010). Multi-year fertility reduction in free-roaming feral horses with single-injection immunocontraceptive formulations. *Wildl. Res.* **37**, 475–481. doi:10.1071/WR09175

Gupta, S. K., and Bansal, P. (2010). Vaccines for immunological control of fertility. *Reprod. Med. Biol.* **9**, 61–71. doi:10.1007/S12522-009-0042-9

Gutierrez, P. L. (2000). The metabolism of quinone-containing alkylating agents: free radical production and measurement. *Front. Biosci.* **5**, D629–D638.

Hampson, B. A., Zabek, M. A., Pollitt, C. C., and Nock, B. (2011). Health and behaviour consequences of feral horse relocation. *Rangeland J.* **33**, 173–180. doi:10.1071/RJ10075

Hanoux, V., Pairault, C., Bakalska, M., Habert, R., and Livera, G. (2007). Caspase-2 involvement during ionizing radiation-induced oocyte death in the mouse ovary. *Cell Death Differ.* **14**, 671–681. doi:10.1038/SJ.CDD.4402052

Hardy, C. M., Hinds, L. A., Kerr, P. J., Lloyd, M. L., Redwood, A. J., Shellam, G. R., and Strive, T. (2006). Biological control of vertebrate pests using virally vectored immunocontraception. *J. Reprod. Immunol.* **71**, 102–111. doi:10.1016/J.JRI.2006.04.006

Herbert, C. A., and Trigg, T. E. (2005). Applications of GnRH in the control and management of fertility in female animals. *Anim. Reprod. Sci.* **88**, 141–153. doi:10.1016/J.ANIREPROSCI.2005.05.007

Hinds, L. (2006). Fertility control of large herbivores: a brief overview of the options. In 'Proceedings of the National Feral Horse Management Workshop,' August 2006, Canberra. (Eds M. J. Dawson, C. Lane and G. Saunders.) pp. 60–64. (Invasive Animals Cooperative Research Centre: Canberra)

Hobbs, N. T., Bowden, D. C., and Baker, D. L. (2000). Effects of fertility control on populations of ungulates: general, stage-structured models. *J. Wildl. Manage.* **64**, 473–491. doi:10.2307/3803245

Hone, J. (1999). On rate of increase(r): patterns of variation in Australian mammals and the implications for wildlife management. *J. Appl. Ecol.* **36**, 709–718. doi:10.1046/J.1365-2664.1999.00439.X

Hoyer, P. B., Devine, P. J., Hu, X., Thompson, K. E., and Sipes, I. G. (2001). Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model. *Toxicol. Pathol.* **29**, 91–99. doi:10.1080/019262301301418892

Humphrys, S., and Lapidge, S. J. (2008). Delivering and registering species-tailored oral antifertility products: a review. *Wildl. Res.* **35**, 578–585. doi:10.1071/WR07145

Hunt, R. J., Claridge, A. W., Fleming, P. J., Cunningham, R. B., Russell, B. G., and Mills, D. J. (2014). Use of an ungulate-specific feed structure as a potential tool for controlling feral goats in Australian forest ecosystems. *Ecol. Manage. Restor.* **15**, 231–238. doi:10.1111/EMR.12129

Imboden, I., Janett, F., Burger, D., Crowe, M., Hässig, M., and Thun, R. (2006). Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare. *Theriogenology* **66**, 1866–1875. doi:10.1016/J.THERIOGENOLOGY.2006.04.038

Jakubowski, H. (2010) The role of paraoxonase 1 in the detoxification of homocysteine thiolactone. In 'Paraoxonases in Inflammation, Infection, and Toxicology'. (Ed. S. T. Reddy.) pp. 113–127. (Springer: Los Angeles.)

Janett, F., Stump, R., Burger, D., and Thun, R. (2009). Suppression of testicular function and sexual behavior by vaccination against GnRH (Equity™) in the adult stallion. *Anim. Reprod. Sci.* **115**, 88–102. doi:10.1016/J.ANIREPROSCI.2008.11.011

Keating, A. F. J., Mark, C., Sen, N., Sipes, I. G., and Hoyer, P. B. (2009). Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7,12-dimethylbenz[a]anthracene

in neonatal rat ovaries. *Toxicol. Appl. Pharmacol.* **241**, 127–134. doi:10.1016/J.TAAP.2009.08.012

Killian, G., Miller, L., Diehl, N., Rhyan, J., and Thain, D. (2006). Long-term efficacy of three contraceptive approaches for population control of wild horses. In 'Proceedings of the 22nd Vertebrate Pest Conference', University of California, Davis. (Eds. R. M. Timm and J. M. O'Brien.) pp. 67–71.

Killian, G., Thain, D., Diehl, N. K., Rhyan, J., and Miller, L. (2008). Four-year contraception rates of mares treated with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices. *Wildl. Res.* **35**, 531–539. doi:10.1071/WR07134

Kim, S.-J., Jung, H.-J., and Lim, C.-J. (2013). Reactive oxygen species-dependent down-regulation of tumor suppressor genes PTEN, USP28, DRAM, TIGAR, and CYLD under oxidative stress. *Biochem. Genet.* **51**, 901–915. doi:10.1007/S10528-013-9616-7

Kirkpatrick, J. F. (2007). Viewpoint: measuring the effects of wildlife contraception: the argument for comparing apples with oranges. *Reprod. Fertil. Dev.* **19**, 548–552. doi:10.1071/RD06163

Kirkpatrick, J. F., and Turner, A. (2002). Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (*Equus caballus*). *Reprod. Suppl.* **60**, 197–202.

Kirkpatrick, J. F., and Turner, A. (2007). Immunocontraception and increased longevity in equids. *Zoo Biol.* **26**, 237–244. doi:10.1002/ZOO.20109

Kirkpatrick, J. F., and Turner, A. (2008). Achieving population goals in a long-lived wildlife species (*Equus caballus*) with contraception. *Wildl. Res.* **35**, 513–519. doi:10.1071/WR07106

Kirkpatrick, J. F., Liu, I. K., and Turner, J. W., Jr (1990). Remotely-delivered immunocontraception in feral horses. *Wildl. Soc. Bull.* **18**, 326–330.

Kirkpatrick, J. F., Turner, J. W., Jr, Liu, I. K., and Fayrer-Hosken, R. (1996). Applications of pig zona pellucida immunocontraception to wildlife fertility control. *J. Reprod. Fertil. Suppl.* **50**, 183–189.

Kirkpatrick, J. F., Lyda, R. O., and Frank, K. M. (2011). Contraceptive vaccines for wildlife: a review. *Am. J. Reprod. Immunol.* **66**, 40–50. doi:10.1111/J.1600-0897.2011.01003.X

Kirkpatrick, J. F., Rutberg, A. T., Coates-Markle, L., and Fazio, P. M. (2012). 'Immunocontraceptive Reproductive Control Utilizing Porcine Zona Pellucida (PZP) in Federal Wild Horse Populations.' (Science and Conservation Center: Billings, MT.)

Klein, C., Scoggin, K. E., Ealy, A. D., and Troedsson, M. H. (2010). Transcriptional profiling of equine endometrium during the time of maternal recognition of pregnancy. *Biol. Reprod.* **83**, 102–113. doi:10.1095/BIOLREPROD.109.081612

Kutzler, M., and Wood, A. (2006). Non-surgical methods of contraception and sterilization. *Theriogenology* **66**, 514–525. doi:10.1016/J.THERIOGENOLOGY.2006.04.014

Lapidge, S. J., Eason, C. T., and Humphrys, S. T. (2010). A review of chemical, biological and fertility control options for the camel in Australia. *Rangeland J.* **32**, 95–115. doi:10.1071/RJ09033

Levin, P. S., Ellis, J., Petrik, R., and Hay, M. E. (2002). Indirect effects of feral horses on estuarine communities. *Conserv. Biol.* **16**, 1364–1371. doi:10.1046/J.1523-1739.2002.01167.X

Liu, I. K. M., Bernoco, M., and Feldman, M. (1989). Contraception in mares heteroimmunized with pig zonae pellucidae. *J. Reprod. Fertil.* **85**, 19–29. doi:10.1530/JRF.0.0850019

Long, J. L. (2003). 'Introduced Mammals of the World: Their History, Distribution and Influence.' (CSIRO Publishing.)

Madosky, J. M., Rubenstein, D. I., Howard, J. J., and Stuska, S. (2010). The effects of immunocontraception on harem fidelity in a feral horse (*Equus caballus*) population. *Appl. Anim. Behav. Sci.* **128**, 50–56. doi:10.1016/J.APPLANIM.2010.09.013

Mask, T. A., Schoenecker, K. A., Kane, A. J., Ransom, J. I., and Bruemmer, J. E. (2015). Serum antibody immunoreactivity to equine zona protein after SpayVac vaccination. *Theriogenology* **84**, 261–267. doi:10.1016/J.THERIOGENOLOGY.2015.03.012

Massei, G., and Cowan, D. (2014). Fertility control to mitigate human–wildlife conflicts: a review. *Wildl. Res.* **41**, 1–21. doi:10.1071/WR13141

Mayer, L. P., Pearsall, N. A., Christian, P. J., Devine, P. J., Payne, C. M., McCuskey, M. K., Marion, S. L., Sipes, I. G., and Hoyer, P. B. (2002). Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. *Reprod. Toxicol.* **16**, 775–781. doi:10.1016/S0890-6238(02)00048-5

McLaughlin, E. A., and Aitken, R. J. (2011). Is there a role for immunocontraception? *Mol. Cell. Endocrinol.* **335**, 78–88. doi:10.1016/J.MCE.2010.04.004

McLaughlin, E. A., and McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137**, 1–11. doi:10.1530/REP-08-0118

McLaughlin, E. A., Holland, M. K., and Aitken, R. J. (2003). Contraceptive vaccines. *Expert Opin. Biol. Ther.* **3**, 829–841. doi:10.1517/14712598.3.5.829

Meirow, D., and Nugent, D. (2001). The effects of radiotherapy and chemotherapy on female reproduction. *Hum. Reprod. Update* **7**, 535–543. doi:10.1093/HUMUPD/7.6.535

Meloen, R. H., Puijk, W. C., and Slootstra, J. W. (2000). Mimotopes: realization of an unlikely concept. *J. Mol. Recognit.* **13**, 352–359. doi:10.1002/1099-1352(200011/12)13:6<352::AID-JMR509>3.0.CO;2-C

Miller, L. A., Gionfriddo, J. P., Fagerstone, K. A., Rhyan, J. C., and Killian, G. J. (2008). The single-shot GnRH immunocontraceptive vaccine (GonaCon^{TM}) in white-tailed deer: comparison of several GnRH preparations. *Am. J. Reprod. Immunol.* **60**, 214–223. doi:10.1111/J.1600-0897.2008.00616.X

Moazamian, R., Polhemus, A., Connaughton, H., Fraser, B., Whiting, S., Gharagozloo, P., and Aitken, R. J. (2015). Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol. Hum. Reprod.* **21**, 502–515. doi:10.1093/MOLEHR/GAV014

Mugnier, S., Dell'Aquila, M. E., Pelaez, J., Douet, C., Ambruosi, B., De Santis, T., Lacalandra, G. M., Lebos, C., Sizaret, P.-Y., and Delaleu, B. (2009). New insights into the mechanisms of fertilization: comparison of the fertilization steps, composition, and structure of the zona pellucida between horses and pigs. *Biol. Reprod.* **81**, 856–870. doi:10.1095/BIOLREPROD.109.077651

Muller, L. I., Warren, R. J., and Evans, D. L. (1997). Theory and practice of immunocontraception in wild mammals. *Wildl. Soc. Bull.* **25**, 504–514.

National Research Council (2013). 'Using Science to Improve the BLM Wild Horse and Burro Program: A Way Forward.' (National Academies Press: Washington, DC.)

Naz, R. K., and Saver, A. E. (2016). Immunocontraception for animals: current status and future perspective. *Am. J. Reprod. Immunol.* **75**, 426–439. doi:10.1111/AJI.12431

Nimmo, D. G., and Miller, K. K. (2007). Ecological and human dimensions of management of feral horses in Australia: a review. *Wildl. Res.* **34**, 408–417. doi:10.1071/WR06102

Nimmo, D. G., Miller, K. K., and Adams, R. (2007). Managing feral horses in Victoria: a study of community attitudes and perceptions. *Ecol. Manage. Restor.* **8**, 237–243. doi:10.1111/J.1442-8903.2007.00375.X

Nixon, B., Aitken, R., and McLaughlin, E. (2007). New insights into the molecular mechanisms of sperm–egg interaction. *Cell. Mol. Life Sci.* **64**, 1805–1823. doi:10.1007/S00018-007-6552-X

Nuñez, C. M., Adelman, J. S., Mason, C., and Rubenstein, D. I. (2009). Immunocontraception decreases group fidelity in a feral horse population during the non-breeding season. *Appl. Anim. Behav. Sci.* **117**, 74–83. doi:10.1016/J.APPLANIM.2008.12.001

Nuñez, C. M., Adelman, J. S., and Rubenstein, D. I. (2010). Immunocontraception in wild horses (*Equus caballus*) extends reproductive cycling

beyond the normal breeding season. *PLoS One* **5**, e13635. doi:10.1371/JOURNAL.PONE.0013635

Paterson, M., Wilson, M. R., Morris, K. D., Duin, M., and Aitken, R. (1998). Evaluation of the contraceptive potential of recombinant human ZP3 and human ZP3 peptides in a primate model: their safety and efficacy. *Am. J. Reprod. Immunol.* **40**, 198–209. doi:10.1111/J.1600-0897.1998.TB00413.X

Powell, D. M., and Monfort, S. L. (2001). Assessment: effects of porcine zona pellucida immunocontraception on estrous cyclicity in feral horses. *J. Appl. Anim. Welf. Sci.* **4**, 271–284. doi:10.1207/S15327604JAWS0404_04

Powers, J. G., Baker, D. L., Davis, T. L., Conner, M. M., Lothridge, A. H., and Nett, T. M. (2011). Effects of gonadotropin-releasing hormone immunization on reproductive function and behavior in captive female Rocky Mountain elk (*Cervus elaphus nelsoni*). *Biol. Reprod.* **85**, 1152–1160. doi:10.1095/BIOLREPROD.110.088237

Powers, J. G., Monello, R. J., Wild, M. A., Spraker, T. R., Gionfriddo, J. P., Nett, T. M., and Baker, D. L. (2014). Effects of GonaCon immunocontraceptive vaccine in free-ranging female Rocky Mountain elk (*Cervus elaphus nelsoni*). *Wildl. Soc. Bull.* **38**, 650–656. doi:10.1002/WSB.434

Prasad, S. V., Skinner, S. M., Carino, C., Wang, N., Cartwright, J., and Dunbar, B. S. (2000). Structure and function of the proteins of the mammalian zona pellucida. *Cells Tissues Organs* **166**, 148–164. doi:10.1159/000016730

Pujianto, D. A., Curry, B. J., and Aitken, R. J. (2010). Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* **151**, 1269–1279. doi:10.1210/EN.2009-0964

Ransom, J. I., Cade, B. S., and Hobbs, N. T. (2010). Influences of immunocontraception on time budgets, social behavior, and body condition in feral horses. *Appl. Anim. Behav. Sci.* **124**, 51–60. doi:10.1016/J.APPLANIM.2010.01.015

Ransom, J. I., Roelle, J. E., Cade, B. S., Coates-Markle, L., and Kane, A. J. (2011). Foaling rates in feral horses treated with the immunocontraceptive porcine zona pellucida. *Wildl. Soc. Bull.* **35**, 343–352. doi:10.1002/WSB.66

Ransom, J. I., Hobbs, N. T., and Bruemmer, J. (2013). Contraception can lead to trophic asynchrony between birth pulse and resources. *PLoS One* **8**, e54972. doi:10.1371/JOURNAL.PONE.0054972

Ransom, J. I., Powers, J. G., Garbe, H. M., Oehler, M. W., Nett, T. M., and Baker, D. L. (2014). Behavior of feral horses in response to culling and GnRH immunocontraception. *Appl. Anim. Behav. Sci.* **157**, 81–92. doi:10.1016/J.APPLANIM.2014.05.002

Reddy, P., Liu, L., Adhikari, D., Jagarlamudi, K., Rajareddy, S., Shen, Y., Du, C., Tang, W., Hämäläinen, T., and Peng, S. L. (2008). Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science* **319**, 611–613. doi:10.1126/SCIENCE.1152257

Roberts, R. M., Xie, S., and Mathialagan, N. (1996). Maternal recognition of pregnancy. *Biol. Reprod.* **54**, 294–302. doi:10.1095/BIOLREPROD54.2.294

Rolls, E. C. (1969). 'They All Ran Wild: The Story of Pests on the Land in Australia.' (Angus and Robertson: Sydney.)

Sabeur, K., Ball, B., Nett, T., Ball, H., and Liu, I. (2003). Effect of GnRH conjugated to pokeweed antiviral protein on reproductive function in adult male dogs. *Reproduction* **125**, 801–806. doi:10.1530/REP.0.1250801

Samoylov, A., Cox, N., Cochran, A., Wolfe, K., Donovan, C., Kutzler, M., Petrenko, V., Baker, H., and Samoylova, T. (2012). Generation and characterization of phage-GnRH chemical conjugates for potential use in cat and dog immunocontraception. *Reprod. Domest. Anim.* **47**(Suppl.), 406–411. doi:10.1111/RDA.12061

Samoylov, A., Cochran, A., Schemera, B., Kutzler, M., Donovan, C., Petrenko, V., Bartol, F., and Samoylova, T. (2015). Humoral immune responses against gonadotropin releasing hormone elicited by immunization with phage-peptide constructs obtained via phage display. *J. Biotechnol.* **216**, 20–28. doi:10.1016/J.JBIOTEC.2015.10.001

Samoylova, T. I., Cox, N. R., Cochran, A. M., Samoylov, A. M., Griffin, B., and Baker, H. J. (2010). ZP-binding peptides identified via phage display stimulate production of sperm antibodies in dogs. *Anim. Reprod. Sci.* **120**, 151–157. doi:10.1016/J.ANIREPROSCI.2010.04.002

Samoylova, T. I., Cochran, A. M., Samoylov, A. M., Schemera, B., Breiteneicher, A. H., Ditchkoff, S. S., Petrenko, V. A., and Cox, N. R. (2012). Phage display allows identification of zona pellucida-binding peptides with species-specific properties: novel approach for development of contraceptive vaccines for wildlife. *J. Biotechnol.* **162**, 311–318. doi:10.1016/J.JBIOTEC.2012.10.006

Schanbacher, B. D., and Pratt, B. R. (1985). Response of a cryptorchid stallion to vaccination against luteinising hormone releasing hormone. *Vet. Rec.* **116**, 74–75. doi:10.1136/VR.116.3.74

Silva, C. A., Yamakami, L. Y. S., Aikawa, N. E., Araujo, D. B., Carvalho, J. F., and Bonfá, E. (2014). Autoimmune primary ovarian insufficiency. *Autoimmun. Rev.* **13**, 427–430. doi:10.1016/J.AUTREV.2014.01.003

Skinner, S. M., Mills, T., Kirchick, H. J., and Dunbar, B. S. (1984). Immunization with zona pellucida proteins results in abnormal ovarian follicular differentiation and inhibition of gonadotropin-induced steroid secretion. *Endocrinology* **115**, 2418–2432. doi:10.1210/ENDO-115-6-2418

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2010). Adding insult to injury: effects of xenobiotic-induced preantral ovotoxicity on ovarian development and oocyte fusibility. *Toxicol. Sci.* **118**, 653–666. doi:10.1093/TOXSCI/KFQ272

Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., and McLaughlin, E. A. (2012). Jumping the gun: smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. *Toxicol. Appl. Pharmacol.* **260**, 70–80. doi:10.1016/J.TAAP.2012.01.028

Spencer, T. E., and Bazer, F. W. (2004). Conceptus signals for establishment and maintenance of pregnancy. *Reprod. Biol. Endocrinol.* **49**, 195–209.

Stout, T. A., and Allen, W. R. (2001). Role of prostaglandins in intrauterine migration of the equine conceptus. *Reproduction* **121**, 771–775. doi:10.1530/REP.0.1210771

Stout, T. A. E., and Colenbrander, B. (2004). Suppressing reproductive activity in horses using GnRH vaccines, antagonists or agonists. *Anim. Reprod. Sci.* **82–83**, 633–643. doi:10.1016/J.ANIREPROSCI.2004.04.009

Swegen, A., and Aitken, R. J. (2016). Prospects for immunocontraception in feral horse population control: exploring novel targets for an equine fertility vaccine. *Reprod. Fertil. Dev.* **28**, 853–863. doi:10.1071/RD14280

Symanski, R. (1994). Contested realities: feral horses in outback Australia. *Ann. Assoc. Am. Geogr.* **84**, 251–269. doi:10.1111/J.1467-8306.1994.TB01737.X

Tung, K. S., Garza, K. M., and Lou, Y.-h. (1999). Induction of pathogenic autoimmune T-cell and autoantibody responses through T-cell epitope mimicry. In 'Autoimmune Reactions'. (Ed. S. Paul) pp. 99–112. (Humana Press: Totowa, NJ)

Tung, K., Agersborg, S., Bagavant, H., Garza, K., and Wei, K. (2002). Autoimmune ovarian disease induced by immunization with zona pellucida (ZP3) peptide. *Curr. Protoc. Immunol.* **15**, 17.

Turkstra, J. A., Van Der Meer, F. J. U. M., Knaap, J., Rottier, P. J. M., Teerds, K. J., Colenbrander, B., and Meloen, R. H. (2005). Effects of GnRH immunization in sexually mature pony stallions. *Anim. Reprod. Sci.* **86**, 247–259. doi:10.1016/J.ANIREPROSCI.2004.07.010

Turner, A., and Kirkpatrick, J. (2002). Effects of immunocontraception on population, longevity and body condition in wild mares (*Equus caballus*). *Reprod. Suppl.* **60**, 187–195.

Turner, J. W., Jr, Liu, I. K. M., Flanagan, D. R., Rutberg, A. T., and Kirkpatrick, J. F. (2001). Immunocontraception in feral horses: one

inoculation provides one year of infertility. *J. Wildl. Manage.* **65**, 235–241. doi:10.2307/3802902

Turner, J. W., Rutberg, A. T., Naugle, R. E., Kaur, M. A., Flanagan, D. R., Bertschinger, H. J., and Liu, I. K. M. (2008). Controlled-release components of PZP contraceptive vaccine extend duration of infertility. *Wildl. Res.* **35**, 555–562. doi:10.1071/WR07159

Wang, G., Li, H., and Firoze Khan, M. (2012). Differential oxidative modification of proteins in $MRL^{+/+}$ and MRL/lpr mice: increased formation of lipid peroxidation-derived aldehyde-protein adducts may contribute to accelerated onset of autoimmune response. *Free Radic. Res.* **46**, 1472–1481. doi:10.3109/10715762.2012.727209

Warren, R. J., Fayrer-Hosken, R. A., Muller, L. I., Willis, L. P., and Goodloe, R. B. (1993). Research and field applications of contraceptives in white-tailed deer, feral horses, and mountain goats. In 'Contraceptive Wildlife Management'. (Ed. J. Wintermute.) 133–145. (UD Department of Agriculture.)

Zabek, M. A., Berman, D. M., Wright, J., Blomberg, S., and Collins, W. C. (2014*a*). Understanding population dynamics of the feral horse in a coniferous environment in southeast Queensland. In 'Proeedings of the 16th Australasian Vertebrate Pest Conference', 2014, Brisbane, Queensland. (Ed. M. Gentle). p. 142.

Zabek, M. A., Wright, J., Berman, D. M., Hampton, J. O., and Collins, C. W. (2014*b*). Assessing the efficacy of medetomidine and tiletamine–zolazepam for remote immobilisation of feral horses (*Equus caballus*). *Wildl. Res.* **41**, 615–622. doi:10.1071/WR14108

Zalba, S. M., and Cozzani, N. C. (2004). The impact of feral horses on grassland bird communities in Argentina. *Anim. Conserv.* **7**, 35–44. doi:10.1017/S1367943003001094

CHAPTER 2

Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) follicular signalling is conserved in the mare ovary

Published: Reproduction, Fertility and Development (2017) 10.1071/RD17024

Authors: Hall SE, Upton RO, McLaughlin EA, Sutherland JM

Chapter 2: Overview

The prospect of developing a non-surgical method of sterilisation by manipulation of local ovarian signalling is promising, given the extensive work which has been done reporting the role and function of each molecule within folliculogenesis cascades in mammals such as mice and humans. The use of a local PTEN inhibitor in the ovary could potentially create an environment which depletes the animal's reproductive potential. However, the signalling pathways responsible for follicular activation in the mare remain uncharacterised. In fact, the anatomical structures of the mare ovary are quite unique. Rather than ovulating at sites within the cortex, the mare ovulates oocytes from an ovulation fossa and these cells subsequently travel to the oviduct. This is potentially an evolutionary adaptation following a process of ovarian involution which occurs during foetal development, where the cortical tissue is invaginated by the medullary region of the ovary.

Little is known about the important precursors that are activated in the horse ovary; however, a deeper understanding of follicular activation in the mare is necessary to determine a path toward permanent fertility control for feral horses. This provided us with the impetus to explore whether the known unique features of horse ovarian anatomy extended to molecular signalling. Specifically, the studies described in this chapter explore mRNA expression and protein localisation of important signalling molecules within the PI3K/AKT and JAK/STAT signalling pathways. We report that mRNA expression of these signalling molecules significantly increased between gestation and adulthood, coincident with sexual maturity. Protein localisation was largely conserved and reflected previous literature on other species. These findings provide insight into what is an elusive reproductive physiology, and may be used as biomarkers to assist in developing a novel technique for non-surgical sterilisation of feral horses.

CSIRO PUBLISHING

Reproduction, Fertility and Development
https://doi.org/10.1071/RD17024

Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) follicular signalling is conserved in the mare ovary

Sally E. Hall[A,B,E], *Rose M. O. Upton*[A], *Eileen A. McLaughlin*[A,C] *and Jessie M. Sutherland*[A,D]

[A]Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, Life Sciences Building, University of Newcastle, Callaghan, NSW 2308, Australia.
[B]Invasive Animals Cooperative Research Centre, Building 22, University of Canberra, Bruce, ACT 2617, Australia.
[C]School of Biological Sciences, Thomas Building, University of Auckland, Auckland 1010, New Zealand.
[D]Pregnancy and Reproduction Program, Hunter Medical Research Institute, New Lambton Heights, NSW 2305, Australia.
[E]Corresponding author. Email: sally.e.hall@uon.edu.au

Abstract. The mare ovary is unique in its anatomical structure; however, the signalling pathways responsible for physiological processes, such as follicular activation, remain uncharacterised. This provided us with the impetus to explore whether signalling molecules from important folliculogenesis pathways, phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and Janus kinase/signal transducer and activator of transcription (JAK/STAT), are conserved in the mare ovary. Messenger RNA expression of six genes important in follicle development was measured using quantitative polymerase chain reaction and protein localisation of key pathway members (PI3K, AKT1, phosphatase and tensin homologue (PTEN), JAK1, STAT3 and suppressor of cytokine signalling 4 (SOCS4)) was compared in tissue from fetal and adult mare ovaries. Tissue from adult ovaries exhibited significantly increased levels of mRNA expression of *PI3K*, *AKT1*, *PTEN*, *JAK1*, *STAT3* and *SOCS4* compared with tissue from fetal ovaries. PI3K, AKT1, JAK1 and STAT3 demonstrated redistributed localisation, from pregranulosa cells in fetal development, to both the oocyte and granulosa cells of follicles in the adult ovary, whilst negative feedback molecules PTEN and SOCS4 were only localised to the granulosa cells in the adult ovary. These findings suggest that the PI3K/AKT and JAK/STAT signalling pathways are utilised during folliculogenesis in the mare, similarly to previously studied mammalian species, and may serve as useful biomarkers for assessment of ovary development in the horse.

Additional keywords: biomarker, folliculogenesis, primordial follicle.

Received 20 January 2017, accepted 3 September 2017, published online 26 September 2017

Introduction

The use of mares in basic physiology research can assist the equine industry in the development of ovarian biomarkers to identify issues associated with horse fertility (Ginther 2012). Similar to other monovular species (Aurich 2011), the mare experiences a considerable 11-month gestation resulting in a single precocial offspring (Platt 1978). Unique to the mare, however, is the anatomical structure of the ovary (Ono *et al.* 2015). Considerably large in size and weight and endowed with a thick connective tissue, the mare ovary has a defined site at which ovulation occurs, referred to as the ovulation fossa (McCue 1998; Aurich 2011; McEntee 2012). Additionally, the ovary has an unusual inverted structure where the medullary tissue is displaced to the outside surface of the ovary. This is potentially due to an evolutionary adaptation following a process of ovarian involution that occurs during fetal development (Sakai 1955; McEntee 2012). It is currently unknown whether the unique features of mare ovarian anatomy extend to molecular signalling.

Primordial follicle activation marks the start of folliculogenesis and requires exquisite control to ensure ongoing and stable reproductive potential (Sutherland *et al.* 2012). Phosphoinositide

3-kinase/protein kinase B (PI3K/AKT; John *et al.* 2008, 2009) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) are signalling pathways highly regarded for their roles in primordial follicle activation (John *et al.* 2008, 2009; Sutherland *et al.* 2010; Sobinoff *et al.* 2013*a*). The PI3K/AKT pathway is well established for its role during initial recruitment in folliculogenesis, with phosphorylation of these molecules essential for primordial follicle survival (Zheng *et al.* 2012). Similarly, previous findings from Sutherland *et al.* (2012) found that members of the JAK/STAT pathway also played a role in primordial follicle regulation in the mouse. Whilst these pathways have been studied extensively in the mouse model they remain completely uncharacterised in the mare. This provided us with the impetus to explore the conservation of important molecules within these pathways.

The aim of this study was to explore the mRNA expression and protein localisation of key signalling molecules in the PI3K/AKT and JAK/STAT pathways from the tissue of fetal filly and adult mare ovaries using quantitative polymerase chain reaction (qPCR) and immunohistochemistry, respectively.

Materials and methods

Materials

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich.

Antibodies

Rabbit polyclonal antibodies to dead-box helicase 4 (VASA; ab12840), JAK1 (ab47435), STAT3 (ab15523), suppressor of cytokine signalling 4 (SOCS4; ab3694), PI3K (ab74136), AKT1 (ab47432), mammalian target of rapamycin (mTOR; ab2732) and forkhead box O3 (FOXO3A; ab47409) were obtained from Abcam. Mouse monoclonal antibody to proliferating cell nuclear antigen (PCNA; 05347) and rabbit polyclonal antibody to phosphatase and tensin homologue (PTEN; 13866) were obtained from Cell Signalling Technology. Rabbit polyclonal antibody to forkhead box L2 (FOXL2; orb100746) was obtained from Biorbyt. Rabbit polyclonal antibody to cardiotrophin-like cytokine factor 1 (CLCF1; 109107) was obtained from GeneTex Inc. Rabbit polyclonal antibody to KIT ligand (KITL; sc9132), stem cell growth factor receptor (KIT; sc5535) and leukaemia inhibitor factor (LIF; sc659) was obtained from Santa Cruz Biotechnology Inc. Rabbit polyclonal antibody to zona pellucida 3 (ZP3) was generated as described previously (Kerr *et al.* 1999). Primary antibody peptide sequences were blasted against *Equus caballus* to ensure it was a conserved domain. Alexa Fluor 594 goat anti-rabbit IgG (A11012) and Alexa Fluor 594 goat anti-mouse IgG (A11005) secondary antibodies were purchased from Invitrogen. For isotype controls, rabbit isotype antibody (31235) was purchased from Thermo Fisher Scientific and mouse IgG (sc2025) and rabbit IgG (sc2027) were obtained from Santa Cruz Biotechnology Inc.

Ovary collection

Horse ovarian tissue was collected from four fetal fillies and four adult mares (fetal $n = 4$, adult $n = 4$) immediately post mortem from a local abattoir and transported to the laboratory on ice within 30 min. Mares were not pregnant at the time of ovary collection. Fetal ovaries were collected from one fetal filly at 3 months gestation, two at 6 months gestation and one at 9 months gestation, estimated by measuring crown–rump length (CRL; Sakai 1955; Bergin 1968; Platt 1978; Franciolli *et al.* 2011). Within 2 h of collection, one ovary from each pair was taken and placed in Bouin's fixative overnight at 4°C, washed three times in 70% ethanol, paraffin embedded and serially sectioned (4 μm), with every fourth slide counterstained with haematoxylin and eosin (H&E). RNAlater (Thermo Fisher Scientific) was immediately added to the remaining ovary according to the manufacturer's instructions and stored at −80°C for protein and RNA analysis.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Sutherland *et al.* 2012). Briefly, slides were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving slides for 9 min in sodium citrate (10 mM, pH 6) or Tris buffer (50 mM, pH 10.6). Slides were cooled to room temperature before being blocked in 3% bovine serum albumin (BSA) Tris-buffered saline (TBS; 10 mM Tris, pH 7.5, 100 mM NaCl) for 1 h. Sections were then incubated with the selected primary antibody (1 : 50–1 : 100 dilution in 1% BSA TBS) overnight at 4°C. After washing three times in TBS containing 0.1% Triton X100 (TBSTx) for 5 min each, sections were incubated with the corresponding fluorescent conjugated secondary antibodies (Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG; 1 : 100 dilution) in 1% BSA TBS for 1 h. Both a positive control (mouse ovary) and negative control (horse adult ovary tested with rabbit or mouse isotype control) were prepared alongside other tissue sections analysed. Additionally, secondary antibody-only controls using mouse ovary, fetal filly and adult mare ovary tissue sections were prepared. All slides were washed three times in TBSTx for 5 min before being counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 1 min. Slides were rinsed in TBS and mounted in antifade reagent Mowiol (13% Mowiol 4–88, 33% glycerol, 66 mM Tris (pH 8.5), 2.5% 1,4 diazobcyclo-[2.2.2]octane). Images were captured on a Zeiss A1 fluorescence microscope. Tissue from the fetal ovary at 3 months gestation is presented in the figures illustrating the ovarian anatomy before involution and hypertrophy.

RNA extraction

RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen). Briefly, ovarian tissue sections from both fetal and adult ovaries were lysed in 350 μL of Buffer RLT using a macerating stick and kept on ice for 10 min. Lysate was centrifuged for 15 min at 13 000*g* at room temperature and the supernatant transferred to a gDNA eliminator spin column and centrifuged for 30 s at 13 000*g* at room temperature. The column was discarded and 350 μL 70% ethanol was added to the flow-through and mixed well by pipetting. The sample was transferred to an RNeasy spin column placed in a collection tube and centrifuged for 15 s at 8000*g* at room temperature. The flow-through was

Table 1. Primer sequences used in mRNA analyses

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphoinositide 3-kinase; AKT1, protein kinase B; PTEN, phosphatase and tensin homologue; JAK1, Janus kinase 1; STAT3, signal transducer and activator of transcription 3; SOCS4, suppressor of cytokine signalling 4

Name	Genebank reference	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Product length	Annealing temp. (°C)
GAPDH	NM_001163856.1	TCAAGCTCATTTCCTGGTAT	CCTTTCTCCTTCTCTTGCT	130	58/55
PI3K	XM_001497768.3	TGCAGCTTAAGATAGGAAGT	TCTTGGTACAGTCCTGAAAA	100	58
AKT1	XM_005605498.1	CAAACACCGTACCATGAAC	GATGACTTCCTTCTTCAGGA	133	55
PTEN	XM_006526769.2	ACAGCCATCATCAAAGAGATCGT	TCAAGTCTTTCTGCAGGAAATCCCA	125	58
JAK1	XM_001918306.2	AACCACATAGCTGATCTGAA	AATACCATTTCCTCCGTCTT	108	58
STAT3	XM_001493633.2	AAATGGGGATTTCTGAGGAT	TTCAGGAATATCTCAGGACG	127	58
SOCS4	NM_001256911.1	TCTCTGTTAGTTTTAGGCGT	CACTTGGGTCCTTATAGTGT	147	55

discarded and 600 µL Buffer RW1 added to the spin column before centrifuging for 15 s at 8000*g* at room temperature. Again the flow-through was discarded and 500 µL Buffer RPE was added to the spin column and centrifuged for 15 s at 10 000*g* at room temperature. Buffer RPE (500 µL) was added to the spin column and centrifuged for 2 min at 8000*g* at room temperature. The RNeasy spin column was placed into a fresh collection tube and 35 µL RNase-free water was added directly to the spin column membrane before centrifuging for 2 min at 8000*g* at room temperature to elute the RNA. The concentration and purity of RNA was assessed using a spectrophotometer, measuring the absorbance at 260 (RNA) and 280 (protein) nm.

Reverse transcription and quantitative PCR

Reverse transcription (RT) was performed as previously described (Sobinoff *et al.* 2013*b*), using 2 µg of isolated RNA, 500 ng oligo(dT)15 primer, 40 U of RNasin, 0.5 mM dNTPs and 20 U of M MLV Reverse Transcriptase (Promega). Reverse transcription reactions were verified by β-actin PCR using cDNA amplified with GoTaq Flexi (Promega). Primer sequences, Genebank references, product length and annealing temperatures are listed in Table 1. Quantitative PCR was performed on cDNA equivalent to 50 ng of total RNA and carried out for 45 amplification cycles using the SYBRGreen GoTaq qPCR master mix (Promega) according to the manufacturer's instructions on a LightCycler 96SW1.0 (Roche). Data was analysed using the equation $2^{-\Delta\Delta C(t)}$, where C(t) is the cycle at which fluorescence was first detected above background fluorescence. Data was normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and presented as the average of each replicate (mean ± s.e.m.).

Statistical analyses

Tissue extractions, RT-PCR and qPCR were conducted in triplicate for all four samples in each age group (fetal and adult). Similarly, all immunofluorescence was performed a minimum of three times across different sections of ovarian tissue from each sample. Data was analysed for normal distribution using the Shapiro–Wilk test for normality in JMP Version 11.2.0 (SAS Institute Inc.). As data were normal, statistical significance was determined using a *t*-test. Graphical data are presented as mean ± s.e.m., the standard errors being calculated from the variance between samples.

Results

Histological analysis of tissue sections from fetal and sexually mature mare ovaries

In the fetal ovary (3 months gestation), H&E staining reveals a clear delineation between the cortex (C) and medulla (M), which, at this timepoint, has not yet undergone involution (Fig. 1*a–b*). Ovigerous cords (OC) can be distinguished running down the medulla to the centre of the ovary (Fig. 1*a*). Oogonial nests (ON) and individual oogonia (O) are observed throughout the tissue (Fig. 1*c*, *e–f*), often surrounding a site of vascularity (Fig. 1*d*, *g–h*). In adult mare ovarian tissue, tissue is primary composed of somatic cells (SC) and luteal cells (LC; Fig. 1*i–j*) with a distinct delineation between cell types. Primordial follicles are sparsely distributed throughout the ovarian stroma consistent with a proliferation of somatic cells within the medulla that occurs late in gestation (Fig. 1*k–o*; McEntee 2012). Primordial follicles are differentiated from primary follicles (Fig. 1*p*) by their flattened squamous epithelial granulosa cells. To confirm these histological observations, immunofluorescence of hallmark proteins on ovarian tissue sections was performed.

Oocyte nests are evident in the fetal ovary and hallmark proteins migrate during development

Immunofluorescence labelling for hallmark ovary-expressed proteins was used in the first instance to assist in identifying the histological arrangement of cell types and structures within the ovary (Fig. 2). Proliferating cell nuclear antigen (PCNA), a marker for follicle proliferation (Hutt *et al.* 2006*b*), was expressed in somatic cell streams undergoing DNA synthesis in the fetal ovary (Fig. 2*a*). PCNA labelling was most apparent in the oocyte nuclei in adult tissue, with lower levels of labelling in surrounding ovarian stroma. Forkhead box protein L2 (FOXL2) is essential for mediating the squamous to cuboidal transition and proliferation of pregranulosa cells during follicle activation (Schmid *et al.* 2004). FOXL2 was indeed labelled in the pregranulosa cells in the fetal ovary consistent with other studies (Sarraj and Drummond 2012) and was found in the granulosa cells as well as the oocyte in the adult ovary (Fig. 2*b*). Dead-box helicase 4 (VASA), a marker for germ-cell development (Sarraj and Drummond 2012), stained oocyte nests, indicative of follicle proliferation. This labelling persisted in adult oocyte cytoplasm (Fig. 2*c*). Finally, zona pellucida 3

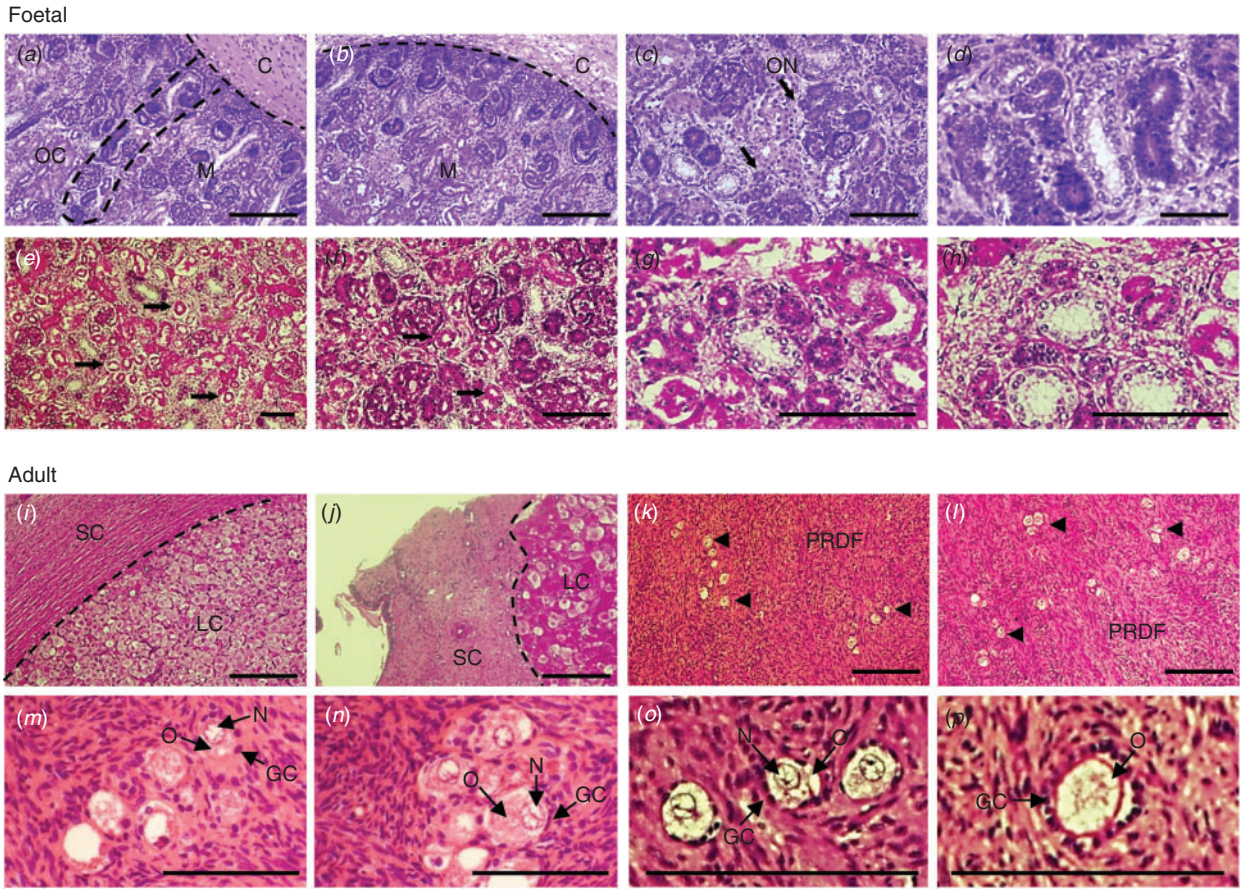


Fig. 1. Histological analysis of tissue sections from (*a–h*) fetal (3 months gestation) and (*i–p*) sexually mature mare ovaries, visualised by H&E staining. (*a*, *b*) In the fetal ovary there is a distinct change in cell structure between the cortex (C) and medulla (M), which has not yet undergone ovarian involution. (*a*) Ovigerous cords (OC) can be distinguished running down the medulla to the centre of the ovary. (*c*, *e–f*) Oogonia (arrows) are present within the tissue and (*d*, *g–h*) the tissue is highly vascular. (*i–j*) In the adult mare ovary, tissue is primary composed of somatic cells (SC) and luteal cells (LC). (*k–l*) Primordial follicles (PRDF, arrow heads) are sparsely located throughout the tissue and are distinguished by (*m–o*) flattened squamous epithelial granulosa cells (GC), oocyte (O) and nucleus (N). (*p*) Primary follicles can be differentiated by cuboidal epithelial granulosa cells (GC). Different tissue regions are delineated by a dotted line. Images are representative of replicates. Scale bar = 100 μM.

(ZP3), a cell membrane and oocyte marker, was detected in the fetal ovary in pregranulosa cells. The expression was different from that observed in adult ovarian tissues, where ZP3 was detected in the oocyte nucleus of follicles (Fig. 2*d*). With an appreciation of the important cellular structures in both fetal and adult tissues, we next went on to assess mRNA expression of important signalling molecules.

PI3K/AKT mRNA expression is significantly higher in the adult mare ovary than in the fetal ovary

Messenger RNA expression, as measured by qPCR, revealed significant increases in important genes within the PI3K/AKT pathway from the time of gestation in the fetal filly to mature development in the adult mare. This rise in mRNA expression is coincident with cyclical follicular activation and oestrus. Messenger RNA of *PI3K* was significantly increased in adult development compared with the fetal ovary ($P < 0.01$; Fig. 3*a*). *AKT1* mRNA expression increased in a similar manner ($P < 0.01$; Fig. 3*b*). Messenger RNA expression of phosphatase and tensin homologue (*PTEN*), the major negative regulator of the PI3K/AKT pathway through dephosphorylation of *AKT1*, also significantly increased from fetal to adult development ($P < 0.01$; Fig. 3*c*). Notably, mRNA expression of *PTEN* was several orders of magnitude higher than *PI3K* and *AKT1*, suggestive of its regulative importance within the pathway. This led us to next assess the protein localisation of these important signalling molecules.

PI3K/AKT protein localisation changes during development in the mare ovary

Localisation of PI3K/AKT signalling molecules PI3K, AKT1 and PTEN was examined via immunofluorescence to determine whether their expression and localisation remained consistent or whether they relocated during development. PI3K, AKT1 and PTEN labelling was localised to pregranulosa cells during fetal development (Fig. 3*d–f*). PI3K labelling was detected in the

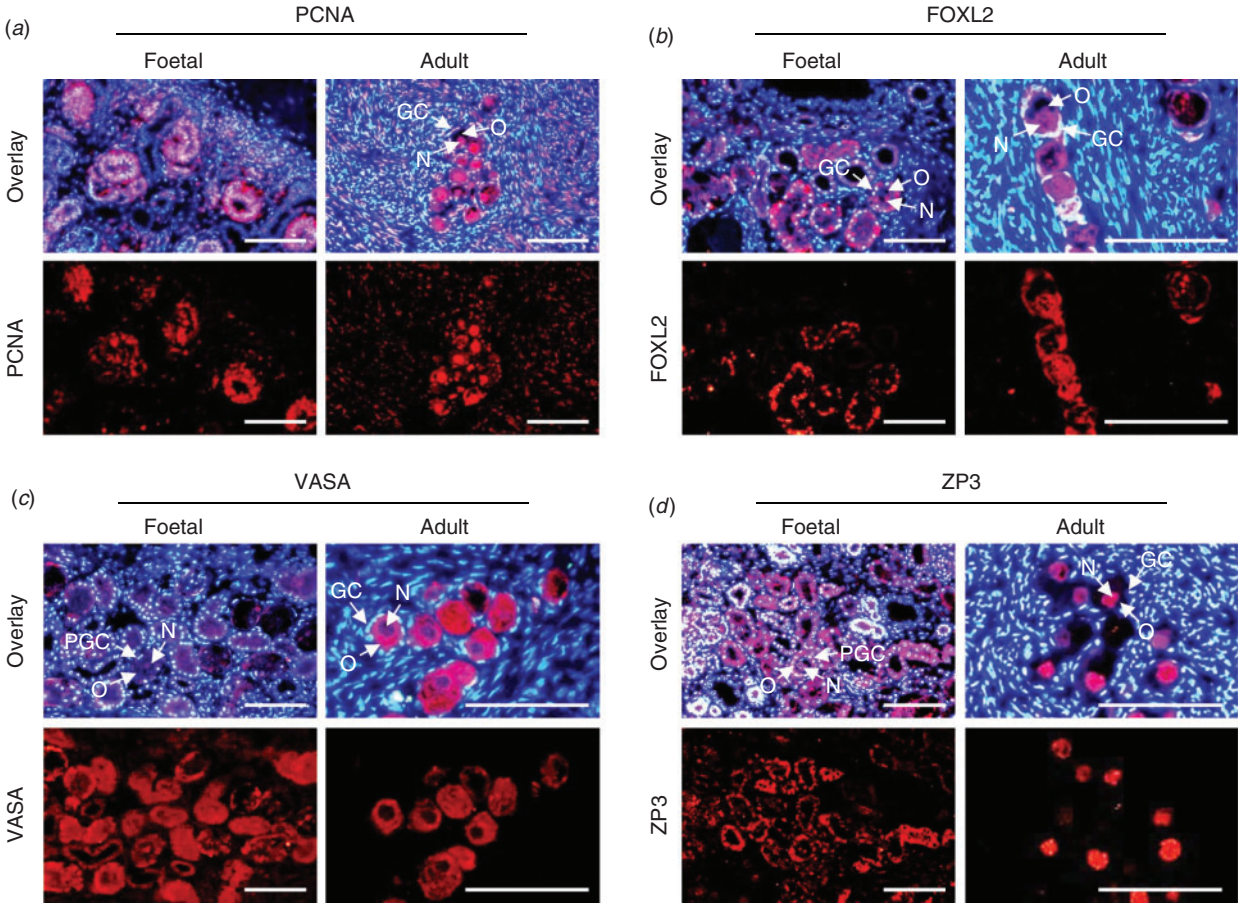


Fig. 2. Hallmark signalling molecules are conserved in the horse ovary, changing localisation during fetal development. Protein localisation of (*a*) PCNA, (*b*) FOXL2, (*c*) VASA and (*d*) ZP3 in the fetal and adult horse ovary, probed with anti-PCNA, -FOXL2, -VASA or -ZP3 and the appropriate fluorescent secondary antibody (Alexa Fluor 594; red). Tissue is counterstained with DAPI (blue). In fetal ovary tissue (3 months gestation) pregranulosa cells (PGC), oogonia (O) and the nucleus (N) are labelled. In the adult mare ovary, primordial follicles are similarly indicated with arrows labelling the oocyte (O), nucleus (N) and granulosa cells (GC). Images are representative of replicates. Scale bar = 100 μM.

cytoplasm of the oocyte in the adult ovary (Fig. 3*d*), consistent with a role in oocyte development. AKT1 protein expression was detected across the entire follicle (comprised of the granulosa cells and oocyte) in the adult ovary (Fig. 3*e*). Finally, PTEN remained localised to the granulosa cells in the adult ovary (Fig. 3*f*).

JAK/STAT mRNA expression is significantly higher in the adult mare ovary than in the fetal ovary

The JAK/STAT molecular signalling pathway was similarly assessed, with a particular focus on *JAK1*, *STAT3* and *SOCS4* gene transcripts (Sutherland *et al.* 2012). *JAK1* mRNA expression was significantly higher in the adult mare ovary compared with the fetal ovary ($P < 0.01$; Fig. 4*a*). The trend of *STAT3* mRNA expression matched *JAK1*, with a significant increase in *STAT3* expression in the adult ovary ($P < 0.01$; Fig. 4*b*). Finally, suppressor of cytokine signalling 4 (*SOCS4*) mRNA expression was also significantly increased between fetal and adult timepoints ($P < 0.05$; Fig. 4*c*). To assess the protein localisation of these signalling molecules, immunofluorescence was performed.

JAK/STAT protein localisation changes during development in the mare ovary

In the fetal ovary, JAK1 was primarily localised to stromal tissue; however, it was primarily redistributed to the granulosa cells in the adult ovary (Fig. 4*d*). In comparison, STAT3 was highly expressed in pregranulosa cells and some oogonia during fetal development and labelled the oocyte and granulosa cells of follicles in the adult ovary (Fig. 4*e*). A diffuse staining pattern for SOCS4 occurred in pregranulosa cells of the fetal ovary and persisted in granulosa cells in the adult ovary (Fig. 4*f*).

Upstream and downstream signalling in the PI3K/AKT and JAK/STAT pathways

Importantly, PI3K/AKT and JAK/STAT are proposed to interact with one another via their upstream receptors, KIT ligand (KITL) and leukaemia inhibitor factor (LIF), respectively (Nilsson *et al.* 2002). Localisation of these receptors was assessed via immunofluorescence (see Fig. S1, available as Supplementary Material to this paper). KITL, KIT and LIF were labelled in pregranulosa cells and migrated into follicles of the

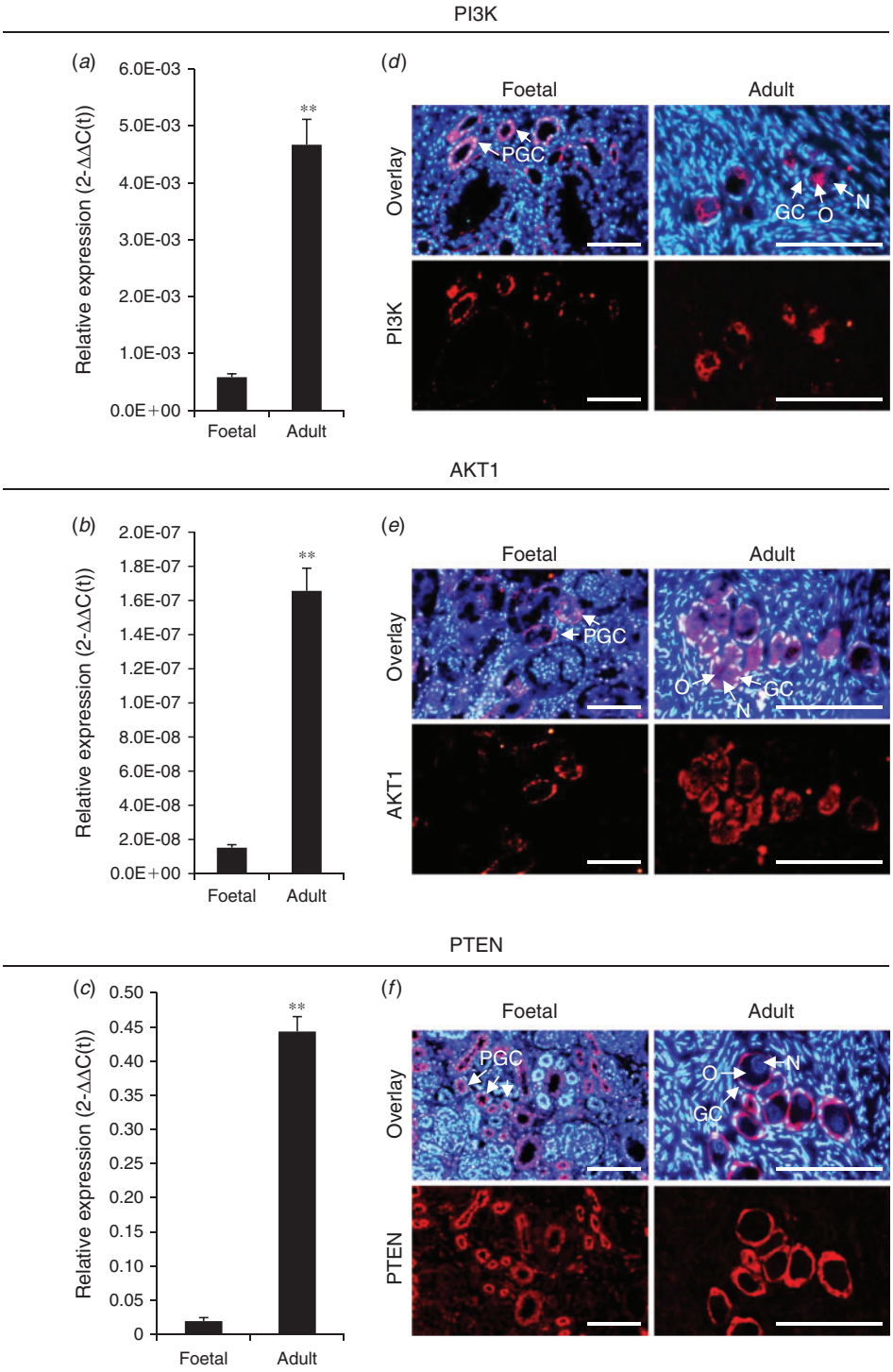


Fig. 3. (*a–c*) Relative mRNA expression of (*a*) *PI3K*, (*b*) *AKT1* and (*c*) *PTEN* between fetal and adult ovaries compared with the reference gene, *GAPDH*. Data correspond to mean values ± s.e.m.; $n = 4$ animals per group. $^{**}P < 0.01$ for significant differences. (*d–f*) Protein localisation of (*d*) PI3K, (*e*) AKT1 and (*f*) PTEN in the fetal and adult horse ovary, probed with anti-PI3K, -AKT1 or -PTEN and the appropriate fluorescent secondary antibody (Alexa Fluor 594; red). Tissue is counterstained with DAPI (blue). Pregranulosa cells (PGC) are labelled in the fetal ovary and granulosa cells (GC), oocyte (O) and nucleus (N) in the adult ovary, indicated by arrows. Images are representative of replicates. Scale bar = 100 μM.

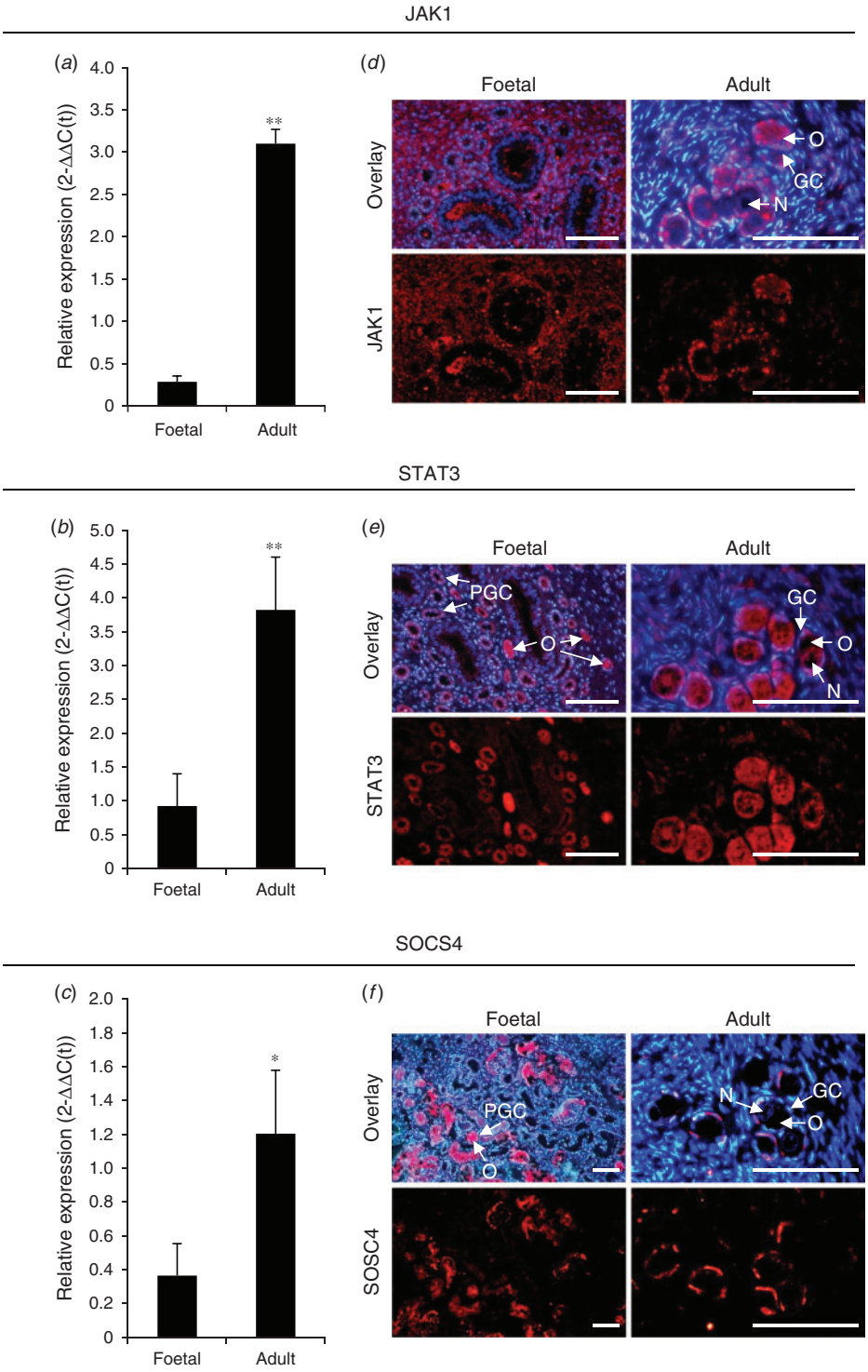


Fig. 4. (*a–c*) Relative mRNA expression of (*a*) *JAK1*, (*b*) *STAT3* and (*c*) *SOCS4* between fetal and adult ovaries compared with the reference gene, *GAPDH*. Data correspond to mean values ± s.e.m.; $n = 4$ animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ for significant differences. (*d–f*) Protein localisation of (*d*) JAK1, (*e*) STAT3 and (*f*) SOCS4 in the fetal and adult horse ovary, probed with anti-JAK1, -STAT3 or -SOCS4 and the appropriate fluorescent secondary antibody (Alexa Fluor 594; red). Tissue is counterstained with DAPI (blue). Pregranulosa cells (PGC) and oogonia (O) are labelled in the fetal ovary and granulosa cells (GC), oocyte (O) and nucleus (N) in the adult ovary, indicated by arrows. Images are representative of replicates. Scale bar = 100 μM.

adult ovary (Fig. S1*a–c*). Upstream JAK/STAT factor CLCF1 labelled oogonia in fetal tissue and was expressed ubiquitously within the adult ovary (Fig. S1*d*). Downstream apoptotic signals were also expressed in the fetal ovary with FOXO3A expression shifting from oogonia in fetal tissue to granulosa cells in the adult ovary (Fig. S1*e*) and mTOR was expressed in pregranulosa cells in the fetal ovary and granulosa cells and oocyte cytoplasm in the adult ovary (Fig. S1*f*).

Discussion

Studies investigating initial recruitment during folliculogenesis have been conducted primarily in the mouse model, reporting the importance of the PI3K/AKT and JAK/STAT pathways in primordial follicle activation (Sutherland *et al.* 2010, 2012; Zheng *et al.* 2012; Wang *et al.* 2016). However, it is unclear whether these findings translate to other species. With variations in ovarian anatomy and reproductive physiology, herein we investigated molecular signalling in the mare ovary during fetal development and at sexual maturity. In this study, two signalling pathways responsible for the onset of folliculogenesis, PI3K/AKT and JAK/STAT, were investigated to determine their conservation within the mare ovary. As these genes are known to be important during primordial follicle activation, this would suggest that higher transcript expression is a requirement during fetal development and that gene transcripts should be higher in younger ovaries. Indeed, transcriptomic analysis of the mouse ovary by Pan *et al.* (2014) reported that genes responsible for primordial follicle activation were higher in neonatal ovaries compared with adult ovaries. However, in the present study, mRNA expression of these genes was detected in both fetal and adult ovarian tissue, as measured via qPCR analysis, with expression levels consistently increased in the adult ovaries when compared with fetal ovaries. These findings are consistent with human ovary studies by Huntriss *et al.* (2017), reporting a significantly higher expression of gene transcripts in sexually mature adult ovaries than fetal ovaries. This was also the case when assessing *KIT* and *KITL* expression in adult versus juvenile rabbits (Hutt *et al.* 2006*a*).

Concerning proteins from the PI3K/AKT signalling pathway, PI3K and AKT1 proteins redistributed from pregranulosa cells in fetal development to the oocyte and granulosa cells of follicles in the adult ovary, whilst PTEN was consistently localised to granulosa cells at both timepoints. The movement in PI3K and AKT1 protein localisation could be the result of changes in the permeability of the nuclear membrane during cellular differentiation, which is what has been reported to occur in mothers against decapentaplegic (SMAD) signalling in the human fetal ovary. Upstream molecules of the PI3K/AKT pathway, KITL and KIT, were localised similarly to the distribution found in human ovarian studies (Childs *et al.* 2010; Tuck *et al.* 2015).

In comparison to PI3K/AKT signalling, little investigation has been done to assess the important molecules that dictate folliculogenesis in the somatic cell JAK/STAT pathway (Sutherland *et al.* 2012; Pastuschek *et al.* 2015; Ndiaye *et al.* 2016). For this reason, we decided to investigate this pathway. JAK1, STAT3 and SOCS4 proteins appear to localise in granulosa cells surrounding the follicle at both timepoints, as was reported in the mouse ovary (Sutherland *et al.* 2012). Interestingly, upstream molecules of this pathway, CLCF1 and LIF, were localised to the oocyte cytoplasm in the adult ovary, rather than granulosa cells as described in the mouse (Sutherland *et al.* 2012). The specific reason behind the differences we see here in protein localisation may be a unique feature of the mare or may be indicative of where these proteins are localised in the ovary of other long-lived species. Future research into this pathway will be necessary to determine this.

There have been extensive studies conducted reporting similarities in follicular waves and dominant follicle selection between the mare and human ovary, reporting the potential for the mare to serve as a model to study ovarian follicular signalling in women (Ginther *et al.* 2004, 2005; Carnevale 2008; Ginther 2012; Schauer *et al.* 2013; Alves *et al.* 2016). Indeed, in the present study, histological analysis of the mare ovary revealed very similar structures to human ovarian tissue, particularly adult ovarian stroma and primordial follicle arrangement (Childs *et al.* 2010; Bayne *et al.* 2015). Furthermore, key hallmark proteins commonly used in human ovarian assessment were successfully observed in mare ovarian tissue. Of particular interest, FOXL2, an important biomarker in the detection of premature ovarian failure in humans (Crisponi *et al.* 2001; Pisarska *et al.* 2004; Uhlenhaut and Treier 2006), was detected in the mare ovary at both fetal and adult timepoints.

Manipulation of these pathways in the mare ovary has also been suggested as a potential strategy in the development of a novel contraceptive for feral horse management (McLaughlin and McIver 2009; Hall *et al.* 2017); however, a lack of characterisation of follicular signalling molecules in the mare ovary has been an impediment in assessing this hypothesis. Loss of function of inhibitory molecules, such as PTEN, and apoptotic signals, such as FOXO3A and mTOR, has been investigated extensively in the literature (Schmid *et al.* 2004; Liu *et al.* 2007; Yoshikawa *et al.* 2007; John *et al.* 2008; Adhikari and Liu 2009; Jagarlamudi *et al.* 2009; Lee *et al.* 2011; McLaughlin *et al.* 2014; Zhang *et al.* 2014; Kim *et al.* 2015) and is reported to cause uncontrolled primordial follicle activation and infertility (Liu *et al.* 2007; Reddy *et al.* 2008; Adhikari and Liu 2009; Adhikari *et al.* 2012). In comparison to *PI3K* and *AKT1* mRNA expression in the mare ovary, *PTEN* mRNA expression was several orders of magnitude higher in the present study. This is suggestive of *PTEN*'s importance in regulating the PI3K/AKT pathway to control the rate at which primordial follicles are activated. Consequently, it is possible that the use of an ovary-specific PTEN inhibitor in the development of a novel contraceptive might activate the primordial follicle pool in the mare ovary and contribute to reproductive senescence, which is reported to occur in this species (Carnevale 2008; Ginther 2012).

In conclusion, this work is the first to provide basic knowledge of these molecules in the mare. These findings provide insight into what has previously been considered an elusive physiology and a framework for future ovary and folliculogenesis studies in this species. There is still much to learn about the molecular mechanisms and genes involved in ovarian development and this is by no means an exhaustive study. Future investigation using a larger sample size and additional timepoints will be required to

further elucidate the findings we report here; however, this may prove difficult due to the limited availability of materials. Nevertheless, the use of mare ovarian tissue offers the opportunity to uncover new findings. Future work will focus on refining our analysis of these signalling molecules in the mare ovary and determining whether these molecules can be used as biomarkers in horse ovary development and fertility.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Grant support: Invasive Animals Cooperative Research Centre (IACRC), project number 2.C.13. Conference presentation: presented in part at the 18th Annual Research Higher Degree Conference, 6 December 2013, Newcastle, NSW, Australia.

References

Adhikari, D., and Liu, K. (2009). Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr. Rev.* **30**(5), 438–464. doi:10.1210/ER.2008-0048

Adhikari, D., Gorre, N., Risal, S., Zhao, Z., Zhang, H., Shen, Y., and Liu, K. (2012). The safe use of a PTEN inhibitor for the activation of dormant mouse primordial follicles and generation of fertilizable eggs. *PLoS One* **7**(6), e39034. doi:10.1371/JOURNAL.PONE.0039034

Alves, K. A., Alves, B. G., Gastal, G. D., de Tarso, S. G., Gastal, M. O., Figueiredo, J. R., Gambarini, M. L., and Gastal, E. L. (2016). The mare model to study the effects of ovarian dynamics on preantral follicle features. *PLoS One* **11**(2), e0149693. doi:10.1371/JOURNAL.PONE.0149693

Aurich, C. (2011). Reproductive cycles of horses. *Anim. Reprod. Sci.* **124**(3–4), 220–228. doi:10.1016/J.ANIREPROSCI.2011.02.005

Bayne, R. A. L., Kinnell, H. L., Coutts, S. M., He, J., Childs, A. J., and Anderson, R. A. (2015). GDF9 is transiently expressed in oocytes before follicle formation in the human fetal ovary and is regulated by a novel NOBOX transcript. *PLoS One* **10**(3), e0119819. doi:10.1371/JOURNAL.PONE.0119819

Bergin, W. C. (1968). 'Developmental Horizons and Measurements Useful for Age Determination of Equine Embryos and Fetuses'. (Kansas State University: Manhattan.)

Carnevale, E. M. (2008). The mare model for follicular maturation and reproductive aging in the woman. *Theriogenology* **69**(1), 23–30. doi:10.1016/J.THERIOGENOLOGY.2007.09.011

Childs, A. J., Kinnell, H. L., Collins, C. S., Hogg, K., Bayne, R. A. L., Green, S. J., McNeilly, A. S., and Anderson, R. A. (2010). BMP signaling in the human fetal ovary is developmentally regulated and promotes primordial germ cell apoptosis. *Stem Cells* **28**(8), 1368–1378. doi:10.1002/STEM.440

Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisceglia, L., Zelante, L., Nagaraja, R., and Porcu, S. (2001). The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat. Genet.* **27**(2), 159–166. doi:10.1038/84781

Franciolli, A. L. R., Cordeiro, B. M., da Fonseca, E. T., Rodrigues, M. N., Sarmento, C. A. P., Ambrosio, C. E., de Carvalho, A. F., Miglino, M. A., and Silva, L. A. (2011). Characteristics of the equine embryo and fetus from days 15 to 107 of pregnancy. *Theriogenology* **76**(5), 819–832. doi:10.1016/J.THERIOGENOLOGY.2011.04.014

Ginther, O. J. (2012). The mare: a 1000-pound guinea pig for study of the ovulatory follicular wave in women. *Theriogenology* **77**(5), 818–828. doi:10.1016/J.THERIOGENOLOGY.2011.09.025

Ginther, O. J., Gastal, E. L., Gastal, M. O., Bergfelt, D. R., Baerwald, A. R., and Pierson, R. A. (2004). Comparative study of the dynamics of follicular waves in mares and women. *Biol. Reprod.* **71**(4), 1195–1201. doi:10.1095/BIOLREPROD.104.031054

Ginther, O. J., Beg, M. A., Gastal, E. L., Gastal, M. O., Baerwald, A. R., and Pierson, R. A. (2005). Systemic concentrations of hormones during the development of follicular waves in mares and women: a comparative study. *Reproduction* **130**(3), 379–388. doi:10.1530/REP.1.00757

Hall, S. E., Nixon, B., and Aitken, R. J. (2017). Non-surgical sterilisation methods may offer a sustainable solution to feral horse (*Equus caballus*) overpopulation. *Reprod. Fertil. Dev.* **29**(9), 1655–1666. doi:10.1071/RD16200

Huntriss, J., Lu, J., Hemmings, K., Bayne, R., Anderson, R., Rutherford, A., Balen, A., Elder, K., and Picton, H. M. (2017). Isolation and expression of the human gametocyte-specific factor 1 gene (GTSF1) in fetal ovary, oocytes, and preimplantation embryos. *J. Assist. Reprod. Genet.* **34**, 23–31. doi:10.1007/S10815-016-0795-0

Hutt, K. J., McLaughlin, E. A., and Holland, M. K. (2006*a*). KIT/KIT ligand in mammalian oogenesis and folliculogenesis: roles in rabbit and murine ovarian follicle activation and oocyte growth. *Biol. Reprod.* **75**(3), 421–433. doi:10.1095/BIOLREPROD.106.051516

Hutt, K. J., McLaughlin, E. A., and Holland, M. K. (2006*b*). Primordial follicle activation and follicular development in the juvenile rabbit ovary. *Cell Tissue Res.* **326**(3), 809–822. doi:10.1007/S00441-006-0223-3

Jagarlamudi, K., Liu, L., Adhikari, D., Reddy, P., Idahl, A., Ottander, U., Lundin, E., and Liu, K. (2009). Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation. *PLoS One* **4**(7), e6186. doi:10.1371/JOURNAL.PONE.0006186

John, G. B., Gallardo, T. D., Shirley, L. J., and Castrillon, D. H. (2008). Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Dev. Biol.* **321**(1), 197–204. doi:10.1016/J.YDBIO.2008.06.017

John, G. B., Shidler, M. J., Besmer, P., and Castrillon, D. H. (2009). Kit signaling via PI3K promotes ovarian follicle maturation but is dispensable for primordial follicle activation. *Dev. Biol.* **331**(2), 292–299. doi:10.1016/J.YDBIO.2009.05.546

Kerr, P. J., Jackson, R. J., Robinson, A. J., Swan, J., Silvers, L., French, N., Clarke, H., Hall, D. F., and Holland, M. K. (1999). Infertility in female rabbits (*Oryctolagus cuniculus*) alloimmunized with the rabbit zona pellucida protein ZPB either as a purified recombinant protein or expressed by recombinant myxoma virus. *Biol. Reprod.* **61**(3), 606–613. doi:10.1095/BIOLREPROD61.3.606

Kim, S. Y., Ebbert, K., Cordeiro, M. H., Romero, M., Zhu, J., Serna, V. A., Whelan, K. A., Woodruff, T. K., and Kurita, T. (2015). Cell autonomous phosphoinositide 3-kinase activation in oocytes disrupts normal ovarian function through promoting survival and overgrowth of ovarian follicles. *Endocrinology* **156**(4), 1464–1476. doi:10.1210/EN.2014-1926

Lee, J.-J., Kim, B. C., Park, M.-J., Lee, Y.-S., Kim, Y.-N., Lee, B. L., and Lee, J.-S. (2011). PTEN status switches cell fate between premature senescence and apoptosis in glioma exposed to ionizing radiation. *Cell Death Differ.* **18**(4), 666–677. doi:10.1038/CDD.2010.139

Liu, L., Rajareddy, S., Reddy, P., Du, C., Jagarlamudi, K., Shen, Y., Gunnarsson, D., Selstam, G., Boman, K., and Liu, K. (2007). Infertility caused by retardation of follicular development in mice with oocyte-specific expression of FOXO3A. *Development* **134**(1), 199–209. doi:10.1242/DEV.02667

McCue, P. M. (1998). Review of ovarian abnormalities in the mare. In 'Proceedings of the Annual Convention of the AAEP, Vol. 44'. pp. 125–33. (Ft. Collins, Colarado)

McEntee, M. (2012). 'Reproductive Pathology of Domestic Mammals'. (Elsevier: San Diego.)

McLaughlin, E. A., and McIver, S. C. (2009). Awakening the oocyte: controlling primordial follicle development. *Reproduction* **137**(1), 1–11. doi:10.1530/REP-08-0118

McLaughlin, M., Kinnell, H. L., Anderson, R. A., and Telfer, E. E. (2014). Inhibition of phosphatase and tensin homologue (PTEN) in human ovary *in vitro* results in increased activation of primordial follicles but compromises development of growing follicles. *Mol. Hum. Reprod.* **20**(8), 736–744. doi:10.1093/MOLEHR/GAU037

Ndiaye, K., Castonguay, A., Benoit, G., Silversides, D. W., and Lussier, J. G. (2016). Differential regulation of Janus kinase 3 (JAK3) in bovine preovulatory follicles and identification of JAK3 interacting proteins in granulosa cells. *J. Ovarian Res.* **9**(1), 71. doi:10.1186/S13048-016-0280-5

Nilsson, E. E., Kezele, P., and Skinner, M. K. (2002). Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol. Cell. Endocrinol.* **188**(1–2), 65–73. doi:10.1016/S0303-7207(01)00746-8

Ono, M., Akuzawa, H., Nambo, Y., Hirano, Y., Kimura, J., Takemoto, S., Nakamura, S., Yokota, H., Himeno, R., Higuchi, T., Ohtaki, T., and Tsumagari, S. (2015). Analysis of the equine ovarian structure during the first twelve months of life by three-dimensional internal structure microscopy. *J. Vet. Med. Sci.* **77**(12), 1599–1603. doi:10.1292/JVMS.14-0539

Pan, L., Gong, W., Zhou, Y., Li, X., Yu, J., and Hu, S. (2014). A comprehensive transcriptomic analysis of infant and adult mouse ovary. *Genomics Proteomics Bioinformatics* **12**(5), 239–248. doi:10.1016/J.GPB.2014.08.002

Pastuschek, J., Poetzsch, J., Morales-Prieto, D. M., Schleußner, E., Markert, U. R., and Georgiev, G. (2015). Stimulation of the JAK/STAT pathway by LIF and OSM in the human granulosa cell line COV434. *J. Reprod. Immunol.* **108**, 48–55. doi:10.1016/J.JRI.2015.03.002

Pisarska, M. D., Bae, J., Klein, C., and Hsueh, A. J. (2004). Forkhead l2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. *Endocrinology* **145**(7), 3424–3433. doi:10.1210/EN.2003-1141

Platt, H. (1978). Growth and maturity in the equine fetus. *J. R. Soc. Med.* **71**(9), 658–661. doi:10.1177/014107687807100906

Reddy, P., Liu, L., Adhikari, D., Jagarlamudi, K., Rajareddy, S., Shen, Y., Du, C., Tang, W., Hämäläinen, T., and Peng, S. L. (2008). Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science* **319**(5863), 611–613. doi:10.1126/SCIENCE.1152257

Sakai, T. (1955). Studies on the development of the embryonic ovary in swine, cattle and horse. *Jpn. J. Vet. Res.* **3**(4), 183–194.

Sarraj, M. A., and Drummond, A. E. (2012). Mammalian foetal ovarian development: consequences for health and disease. *Reproduction* **143**(2), 151–163. doi:10.1530/REP-11-0247

Schauer, S. N., Sontakke, S. D., Watson, E. D., Esteves, C. L., and Donadeu, F. X. (2013). Involvement of miRNAs in equine follicle development. *Reproduction* **146**(3), 273–282. doi:10.1530/REP-13-0107

Schmid, A. C., Byrne, R. D., Vilar, R., and Woscholski, R. (2004). Bisperoxovanadium compounds are potent PTEN inhibitors. *FEBS Lett.* **566**(1–3), 35–38. doi:10.1016/J.FEBSLET.2004.03.102

Sobinoff, A. P., Sutherland, J. M., and Mclaughlin, E. A. (2013*a*). Intracellular signalling during female gametogenesis. *Mol. Hum. Reprod.* **19**(5), 265–278. doi:10.1093/MOLEHR/GAS065

Sobinoff, A. P., Beckett, E. L., Jarnicki, A. G., Sutherland, J. M., McCluskey, A., Hansbro, P. M., and McLaughlin, E. A. (2013*b*). Scrambled and fried: cigarette smoke exposure causes antral follicle destruction and oocyte dysfunction through oxidative stress. *Toxicol. Appl. Pharmacol.* **271**(2), 156–167. doi:10.1016/J.TAAP.2013.05.009

Sutherland, J. M., Keightley, R., Robker, R. L., Russell, D. L., and McLaughlin, E. A. (2010). JAK/STAT signalling in folliculogenesis. *Reprod. Fertil. Dev.* **22**(9), 44. doi:10.1071/SRB10ABS126

Sutherland, J. M., Keightley, R. A., Nixon, B., Roman, S. D., Robker, R. L., Russell, D. L., and McLaughlin, E. A. (2012). Suppressor of cytokine signaling 4 (SOCS4): moderator of ovarian primordial follicle activation. *J. Cell. Physiol.* **227**(3), 1188–1198. doi:10.1002/JCP.22837

Tuck, A. R., Robker, R. L., Norman, R. J., Tilley, W. D., and Hickey, T. E. (2015). Expression and localisation of c-kit and KITL in the adult human ovary. *J. Ovarian Res.* **8**(1), 31. doi:10.1186/S13048-015-0159-X

Uhlenhaut, N. H., and Treier, M. (2006). Foxl2 function in ovarian development. *Mol. Genet. Metab.* **88**(3), 225–234. doi:10.1016/J.YMGME.2006.03.005

Wang, L.-Q., Liu, J.-C., Chen, C.-L., Cheng, S.-F., Sun, X.-F., Zhao, Y., Yin, S., Hou, Z.-M., Pan, B., Ding, C., Shen, W., and Zhang, X.-F. (2016). Regulation of primordial follicle recruitment by cross-talk between the Notch and phosphatase and tensin homologue (PTEN)/AKT pathways. *Reprod. Fertil. Dev.* **28**(6), 700–712. doi:10.1071/RD14212

Yoshikawa, K., Nigorikawa, K., Tsukamoto, M., Tamura, N., Hazeki, K., and Hazeki, O. (2007). Inhibition of PTEN and activation of Akt by menadione. *Biochim. Biophys. Acta* **1770**(4), 687–693. doi:10.1016/J.BBAGEN.2006.12.009

Zhang, H., Risal, S., Gorre, N., Busayavalasa, K., Li, X., Shen, Y., Bosbach, B., Brannstrom, M., and Liu, K. (2014). Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice. *Curr. Biol.* **24**(21), 2501–2508. doi:10.1016/J.CUB.2014.09.023

Zheng, W., Nagaraju, G., Liu, Z., and Liu, K. (2012). Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary. *Mol. Cell. Endocrinol.* **356**(1–2), 24–30. doi:10.1016/J.MCE.2011.05.027

CHAPTER 3

Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa

Published: Biology of Reproduction (2017)

10.1095/biolreprod.116.145292

Authors: Hall SE, Aitken RJ, Nixon B, Smith ND, Gibb Z

Chapter 3: Overview

Given the ready availability of stallion spermatozoa, we next moved our focus toward spermatozoa, which unlike female tissue, offers contraceptive coverage for both sexes by targeting sperm proteins. Stallion spermatozoa are exposed to approximately 32 times the level of ROS during normal metabolism, compared to glycolysis-dependent species such as the human and mouse, due to their reliance on oxidative phosphorylation (OXPHOS) for the majority of their energy demands. This leaves stallion spermatozoa highly susceptible to the generation of downstream products of lipid peroxidation, electrophilic aldehydes, which can form covalent modifications on proteins important for fertilisation.

Prior to investigating whether such modifications could be exploited to enhance the immunogenicity of sperm proteins, the aim of the studies conducted in this chapter was to firstly demonstrate that the electrophilic aldehydes ACR, 4HNE and malondialdehyde (MDA) can adduct to stallion sperm proteins of functional significance. For this purpose, stallion spermatozoa exposed to exogenous aldehydes were assessed for the presence of a detoxification mechanism within the mitochondria, perturbation of the physiological characteristics of the cell, and identification of the complement of vulnerable proteins targeted for adduction.

In this chapter, we report a significant increase in mitochondrial and cytosolic ROS, lipid peroxidation and a commensurate loss of motility following exposure to ACR and 4HNE. ALDH2 appeared responsible for counteracting acute ROS formation; however, its activity was overwhelmed by 24 h exposure. HSP90AA1 and ARSA were sites vulnerable to oxidative modification by ACR and 4HNE, respectively, subsequently resulting in loss of zona pellucida binding competence.

The work in this chapter therefore provides proof-of-concept that stallion sperm proteins are vulnerable to adduction by electrophilic aldehydes. Further, these

proteins include representatives that are likely of key functional importance for achieving conception, and therefore provide the impetus for additional investigation into the utility of using this approach in contraceptive studies to inhibit sperm-oocyte interactions (as reported in Chapter 4).

Biology of Reproduction, 2017, 00(0), 1–15
doi:10.1095/biolreprod.116.145292
Research Article

OXFORD

Research Article

Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa†

Sally E. Hall[1,2,*], R. John Aitken[1,2], Brett Nixon[1], Nathan D. Smith[3] and Zamira Gibb[1]

[1]Priority Research Centre for Reproductive Science, Discipline of Biological Sciences, Faculty of Science and Information Technology, University of Newcastle, Callaghan, New South Wales, Australia; [2]Invasive Animals Cooperative Research Centre, University of Canberra, Bruce, Australian Capital Territory, Australia and [3]Analytical and Biomedical Research Facility, Research Division, University of Newcastle, Callaghan, New South Wales, Australia

*Correspondence: Priority Research Centre for Reproductive Science, Level 4, Life Sciences Building, Ring Road, Discipline of Biological Sciences, Faculty of Science and Information Technology, University of Newcastle, Callaghan, NSW, 2308, Australia. Email: sally.e.hall@uon.edu.au

†Supported by Invasive Animals Cooperative Research Centre (#2.C.13) and Australian Research Council Linkage Grant (LP120100219).
Presented in part at the 46th annual meeting of the Society for Reproductive Biology, 23–26 August 2015, Adelaide, South Australia and at the 18th International Congress on Animal Reproduction, 26–30 June 2016, Tours, France.

Received 30 September 2016; Revised 10 November 2016; Accepted 28 November 2016

Abstract

Oxidative stress is a major determinant of mammalian sperm function stimulating lipid peroxidation cascades that culminate in the generation of potentially cytotoxic aldehydes. The aim of this study was to assess the impact of such aldehydes on the functionality of stallion spermatozoa. The impact of exposure to exogenous acrolein (ACR) and 4-hydroxynonenal (4HNE) was manifested in a highly significant dose- and time-dependent increase in mitochondrial reactive oxygen species (ROS), total cellular ROS, a decrease in sperm motility, and a time-dependent increase in lipid peroxidation. Notably, low doses of ACR and 4HNE also caused a significant decrease in zona binding. In contrast, exogenous malondialdehyde, a commonly used marker of oxidative stress, had little impact on the various sperm parameters assessed. In accounting for the negative physiological impact of ACR and 4HNE, it was noted that both aldehydes readily adducted to sperm proteins located predominantly within the head, proximal centriole, and tail. The detoxifying activity of mitochondrial aldehyde dehydrogenase 2 appeared responsible for a lack of adduction in the midpiece; however, this activity was overwhelmed by 24 h of electrophilic aldehyde exposure. Sequencing of the dominant proteins targeted for ACR and 4HNE covalent modification identified heat shock protein 90 alpha (cytosolic) class A member 1 and arylsulfatase A, respectively. These collective findings may prove useful in the identification of diagnostic biomarkers of stallion fertility and resolving the mechanistic basis of sperm dysfunction in this species.

Summary Sentence

ALDH2 activity is exacerbated following electrophilic aldehyde exposure, and HSP90AA1 and ARSA are vulnerable sites for adduction.

Key words: 4-hydroxynonenal, acrolein, ARSA, aldehyde dehydrogenase, electrophilic aldehydes, HSP90AA1, lipid peroxidation, oxidative stress, reactive oxygen species, spermatozoa, stallion.

Introduction

Oxidative stress is due to a fundamental imbalance between the generation of reactive oxygen species (ROS) and the buffering capacity of antioxidant enzymes and free radical scavengers within the cell [1–4]. In the case of mammalian spermatozoa, this self-perpetuating cascade [5] has been shown to elicit lipid peroxidation and the downstream production of a suite of reactive electrophilic aldehydes, which have the capacity to form covalent adducts with macromolecules such as DNA and protein, ultimately resulting in the perturbation of cell integrity and function [6–9]. The sperm cell is widely regarded as being unique in its extreme vulnerability to lipid peroxidation, owing to the presence of high levels of polyunsaturated fatty acids (PUFAs) within the plasma membrane [4,7,10–13], as well as transition metals that perpetuate the oxidative stress cycle by acting as a catalyst for the Fenton reaction [14,15].

However, it has recently been demonstrated that the spermatozoa of all mammalian species are not equally susceptible to this form of oxidative insult. Indeed, unlike the spermatozoa of humans and mice, those of the stallion experience high levels of oxidative stress owing to their reliance on oxidative phosphorylation (OXPHOS) for the generation of the majority of their energy demands [16,17]. In principle, this strategy should exacerbate the oxidative stress cascade and render stallion spermatozoa at increased risk of oxidative lesions. Although it is true that stallion spermatozoa are highly metabolically active and short-lived cells [6,16], they nonetheless appear to have co-opted a set of defense mechanisms to counteract the damaging impact of endogenous ROS production. Commonly referred to as ROS homeostasis [18], defenses against oxidative stress include the relatively high concentrations of antioxidants such as catalase [19], superoxide dismutase [2,19–21], glutathione [18,22–25], and ergothioneine [26] that are present in stallion semen. Similarly, these cells also draw on mitochondrial aldehyde dehydrogenase 2 (ALDH2) to act as a detoxifier of aldehydes generated either endogenously or exogenously [27].

The aim of this study was to explore the vulnerability of stallion spermatozoa to the impact of electrophilic aldehydes by comparing the effects of exogenous acrolein (ACR), 4-hydroxynonenal (4HNE), and malondialdehyde (MDA) on stallion sperm functionality. In addition, in view of the emerging importance of ALDH2 in the primary defense of stallion sperm functionality during oxidative stress events, we hypothesized that this enzyme would play a pivotal role in ameliorating the impact of an acute electrophilic aldehyde challenge. Finally, to provide mechanistic insight to account for any loss of function, we also assessed the capacity of these electrophilic aldehydes to form adducts with key functional proteins represented in the stallion sperm proteome [28], and assessed zona binding competence.

Materials and methods

Experimental design

This study was designed to identify protein targets susceptible to adduction following exposure of stallion spermatozoa to the electrophilic aldehydes ACR, 4HNE, and MDA. Initial experiments focused on the assessment of oxidative stress parameters including mitochondrial ROS, cytosolic ROS, and lipid peroxidation, and consequent changes to motility and vitality at intervals of 3 and 24 h after electrophilic aldehyde exposure. For the purpose of these studies, sperm motility was objectively determined using computer-assisted sperm analysis and the percentage of live cells in a given population (i.e. sperm vitality) was assessed via differential fluorophore labeling, as outlined below. Immunocytochemical analysis revealed a lack of aldehyde adduction at the site of the midpiece, which was further investigated by examining the distribution of ALDH, an enzyme that has previously been implicated in the protection of stallion spermatozoa from oxidative insult by virtue of its ability to metabolize aldehydes as they accumulate within the mitochondria [29]. To examine the potential role of ALDH in the detoxification of electrophilic aldehydes within this cellular domain, its enzymatic activity was inhibited by treating cells with aldehyde dehydrogenase inhibitor 2 (ALDI2) prior to exposure to electrophilic aldehydes.

In view of the ability of ACR or 4HNE treatments to elicit potent oxidative stress responses in stallion spermatozoa, we subsequently undertook immunoblotting and mass spectrometry analyses to identify those proteins targeted for selective adduction by either of these reactive aldehydes. To determine the identity of the prominent targets for ACR and 4HNE adduction, both the 90 and 60 kDa bands were excised and subjected to nanoliquid chromatography- tandem mass spectrometry (LC–MS/MS) using reversed-phase LC coupled to an electrospray ionization 3D ion trap mass spectrometer interface. The validity of identified protein targets was then established via immunocytochemical and immunoprecipitation (IP) strategies. For this purpose, sperm lysates prepared from ACR-treated stallion spermatozoa were either pulled down with anti-ACR or anti-heat shock protein 90 alpha (cytosolic) class A member 1 (HSP90AA1), and the corresponding eluates assessed for both HSP90AA1 and ACR. Similarly, IP of sperm lysates from 4HNE-treated stallion spermatozoa was either pulled down with anti-4HNE or anti-arylsulfatase A (ARSA), and the corresponding eluates assessed for both ARSA and 4HNE. Furthermore, the identity of ACR and 4HNE adducted proteins led us to investigate whether exposure to these reactive aldehydes could affect sperm-zona binding competence independent of a concomitant reduction in sperm motility. For this purpose, we employed an established heterologous sperm-zona binding assay in which ACR and 4HNE-treated stallion spermatozoa were assessed for their adhesion to bovine oocytes [30–32].

Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). A modified Biggers, Whitten, and Whittingham (BWW) medium containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 275 μM sodium pyruvate, 3.7 μl/ml 60% sodium lactate syrup, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.1% (w/v) polyvinyl alcohol, with an osmolarity between 290 and 310 mOsm/kg and a

Table 1. Antibodies used throughout the duration of the study

Antibody	Raised	Catalogue	Distributor
Acrolein	Rabbit	NB200-556	Novus Biologicals
4-hydroxynonenal	Rabbit	HNE11-S	Jomar Diagnostics
4-hydroxynonenal	Mouse	ab48506	Abcam
Malondialdehyde	Rabbit	ab6463	Abcam
heat shock protein 90 alpha (cytosolic) class A member 1	Mouse	H38220	BD Transduction Laboratories
Arylsulfatase A	Rabbit	HPA005554	Sigma-Aldrich

pH of approximately 7.4, was used for stallion sperm incubations throughout this study [33]. For incubation periods exceeding 12 h, BWW was further supplemented with gentamicin at 0.25 mg/ml to restrict the growth of *Pseudomonas aeruginosa* [34].

Antibodies

Rabbit polyclonal anti-ACR was obtained from Novus Biologicals (NB200-556; Littleton, CO, USA). Rabbit polyclonal anti-4HNE was obtained from Jomar Diagnostics (HNE11-S; Adelaide, SA, Australia). Mouse polyclonal anti-4HNE (ab48506) and rabbit polyclonal anti-MDA (ab6463) were obtained from Abcam (Melbourne, VIC, Australia). Mouse monoclonal antibody to HSP90AA1 was obtained from BD Transduction Laboratories (H38220; North Ryde, NSW). Rabbit polyclonal antibody to ARSA was obtained from Sigma-Aldrich (HPA005554; Castle Hill).

Animal ethics

Institutional and New South Wales State Government ethical approval was secured for the use of animal materials in this study (#A-2011-122). This research was based on multiple donations from three normozoospermic Miniature and Shetland cross stallions of proven fertility.

Preparation of spermatozoa

Stallion spermatozoa were collected using a pony-sized Missouri artificial vagina (Minitube Australia, Ballarat, VIC). Ejaculates were immediately diluted (1:2, semen:extender) with Kenney extender consisting of 272 mM glucose, 24 mg/ml skim milk powder, 1500 U/ml penicillin, and 1.5 mg/ml streptomycin [35]. Equipment and extender were maintained at temperatures between 30°C and 37°C for the duration of semen collection and dilution. Extended semen was transported to the laboratory within 1 h of collection in a polystyrene box at room temperature (RT). On arrival at the laboratory, high-quality spermatozoa were isolated from extended semen using a discontinuous Percoll gradient (centrifuged at 500 × *g* for 30 min) as previously described [36]. The isolated sperm pellet was recovered from the base of the 80% fraction of the gradient, resuspended to a volume of 10 ml in BWW medium, and washed via centrifugation at 500 × *g* for 15 min. Sperm concentration was determined using a NucleoCounter SP-100 (ChemoMetec, Allerod, Denmark) and diluted to a final concentration of 20 × 10^6 spermatozoa/ml in BWW for the duration of the experiments.

ACR, 4HNE, and MDA were used to assess the time-dependent (3 and 24 h) and dose-dependent (0, 50, 100, and 200 μM) effects of electrophilic aldehyde exposure on stallion spermatozoa. The doses chosen mirror pathological physiological ranges (30–180 μM ACR, 10 μM–5 mM 4HNE) [37–40], and selected time points correspond with previous studies conducted on human spermatozoa [7,41]. Cells were washed in BWW (100 μl aliquots, 3 min at 500 × *g*) prior to performing the functional assays described below to remove free aldehydes from solution.

Motility analysis

Sperm motility was objectively determined using computer-assisted sperm analysis (IVOS, Hamilton Thorne, Danvers, MA, USA) employing the following settings: negative phase-contrast optics, recording rate of 60 frames/s, minimum contrast of 70, minimum cell size of four pixels, low size gate of 0.17, high size gate of 2.9, low-intensity gate of 0.6, high-intensity gate of 1.74, nonmotile head size of 10 pixels, nonmotile head intensity of 135, progressive average path velocity (VAP) threshold of 50 μm/s, slow (static) cell VAP threshold of 20 μm/s, slow (static) cell velocity (VSL) threshold of 0 μm/s, and threshold straightness (STR) of 75% [16]. Cells exhibiting a VAP of $\geq$ 50 μm/s and an STR of $\geq$ 75% were considered progressive. A minimum of 200 spermatozoa in a minimum of five fields were assessed using 20 μm Leja standard count slides (Gytech, Hawthorn East, VIC) and a stage temperature of 37°C.

Flow cytometry

Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with 488 and 635 nm argon ion lasers. Emission measurements were made using 530/30 bandpass (green/FL-1), 585/42 bandpass (red/FL-2), and > 670 longpass (far red/FL-4) filters. Debris was gated out using a forward scatter/side scatter dot plot, and a minimum of 5000 cells were analyzed per sample. The position of the positive controls on the dot plots was used to set the gates between positive and negative populations. All data were analyzed using the CellQuest Pro software (Becton Dickinson). To measure mitochondrial and total cellular ROS, MitoSOX Red (MSR; Thermo Fisher Scientific, Scoresby, VIC) and dihydroethidium (DHE; Thermo Fisher Scientific) were used, respectively, with results reported as the percentage of live, positive cells, as previously described [41,42]. Briefly, 100 μl of each sperm suspension was incubated at 37°C for 15 min with respective probes reconstituted according to the manufacturer's instructions at final concentrations of 2 μM MSR or DHE using 0.1 μl/ml green LIVE/DEAD (Thermo Fisher Scientific) as a vitality stain. Following incubation, spermatozoa were centrifuged at 500 × *g* for 3 min and the pellet was resuspended in BWW for flow cytometric analysis. For both MSR and DHE assessments, an unstained control, a MSR or DHE-positive control (using a final concentration of 25 μM arachidonic acid added at the same time as the probe), and a snap-frozen LIVE/DEAD positive control were included in each replicate [41,43].

To measure lipid peroxidation, spermatozoa were preloaded with 5 μM BODIPY C11 (Thermo Fisher Scientific) for 30 min at 37°C, as described previously [14]. Sperm were washed with BWW (500 × *g* for 3 min), resuspended in BWW, and treated with respective aldehydes. At 3 and 24 h time points, these cells were washed to remove unbound aldehydes and were incubated with 0.1 μl/ml far red LIVE/DEAD vitality stain (Thermo Fisher Scientific) with live cells being gated for BODIPY C11 analysis. Cells were categorized as having a positive or negative BODIPY C11 signal, with the gate between high and low lipid peroxidation being determined using an 80 μM ferrous sulfate positive control (added 1 h prior to flow cytometric analysis) [43].

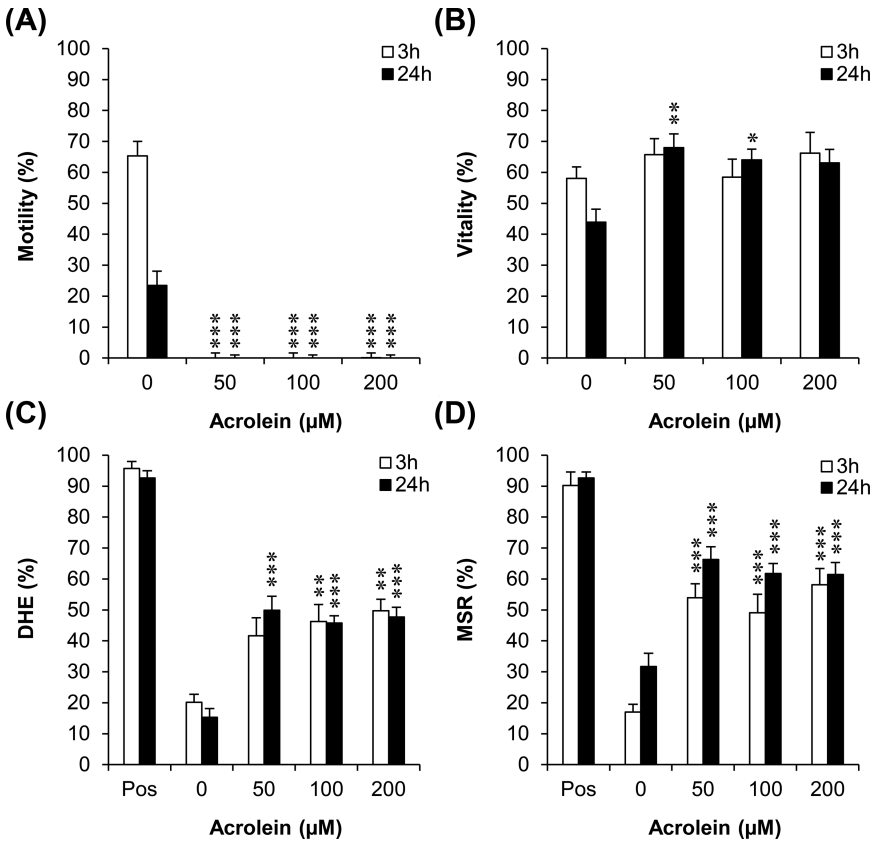


Figure 1. **A**) Motility, **B**) vitality, **C**) total cellular ROS, and **D**) mitochondrial ROS generation following a dose- and time-dependent exposure to ACR at 37°C. Positive control (Pos) generated by exposing the same populations of spermatozoa to arachidonic acid (50 μM). Data correspond to mean values ± SEM; n = 9 independent ejaculates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences compared with untreated control sample.

Immunocytochemistry

To assess aldehyde surface labeling, live spermatozoa were incubated in a 1:50 dilution of anti-ACR antibody, anti-4HNE antibody, or anti-MDA antibody in BWW in the dark for 30 min at 37°C. Cells were washed three times in BWW and incubated with goat-anti-rabbit Alexa Fluor 488 secondary antibody (1:400) in the dark for 15 min at 37°C. Secondary antibody only controls were used to monitor nonspecific binding, and Hoechst 33258 was used as a vitality stain (1:1000).

Measurement of ALDH2 activity

ALDH2 activity was assessed in all 50 μM aldehyde treatments using the Aldefluor assay kit (StemCell Technologies, Tullamarine, VIC). Live spermatozoa were centrifuged at 500 × *g* for 3 min and washed in BWW, then resuspended in Aldefluor buffer containing 5 μl/ml activated Aldefluor reagent for 30 min at 37°C as per the manufacturer's instructions. To assess aldehyde localization following the inhibition of ALDH, cells were incubated in 10 μM ALDI2 [44] for 10 min before being exposed to the respective 50 μM aldehyde treatments. Spermatozoa were then centrifuged, washed, and incubated in Aldefluor buffer for 30 min at 37°C, as mentioned previously. An ALDI2-treated (ALDH inhibited) sperm sample that was not exposed to any aldehydes was also stained with Aldefluor reagent and this acted as the negative control. Following staining, spermatozoa were centrifuged at 500 × *g* for 3 min and washed three times in Aldefluor buffer and mounted on slides for imaging.

To compare 3 and 24 h time points, and for colocalization imaging, spermatozoa were centrifuged at 500 × *g* for 3 min, washed three times in BWW, and fixed with 2% paraformaldehyde in phosphate-buffered saline for 5 min at 4°C. Cells were then washed three times in 0.1 M glycine in PBS. Cells were permeabilized for 5 min at 4°C in solution consisting of 3.5 mM sodium citrate and 0.1% Triton X-100 in PBS. Cells were then centrifuged at 500 × *g* for 3 min and washed three times in PBS. Spermatozoa were blocked in 3% bovine serum albumin (BSA)/10% goat serum in PBS for 1 h at RT, and primary antibodies against each aldehyde or protein (1:50) applied in 1% BSA in PBS overnight at RT. Cells were washed three times in PBS and centrifuged at 500 × *g* for 3 min and secondary antibodies (anti-mouse, anti-rabbit; 1:200) applied in 1% BSA in PBS for 1 h at RT. Finally, cells were centrifuged at 500 × *g* for 3 min and washed three times in PBS. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), washed, and mounted. Images were captured on an Axio Imager A1 fluorescence microscope (Zeiss, North Ryde, NSW) using cellSens Standard imaging software (Olympus, Notting Hill, VIC).

Gel electrophoresis and immunoblotting

Cell lysates were prepared from sperm pellets (~ 50 × 10^6 spermatozoa) following treatment with each aldehyde (50 μM, 3 h incubation at 37°C) by resuspending pellets in 50 μl 10 mM CHAPS lysis buffer with the addition of protease inhibitors (Roche Diagnostics, Castle Hill, NSW) for 1 h at 4°C with constant rotation and vortexing every 10 min. The cell suspension was then centrifuged at 14,000 × *g*

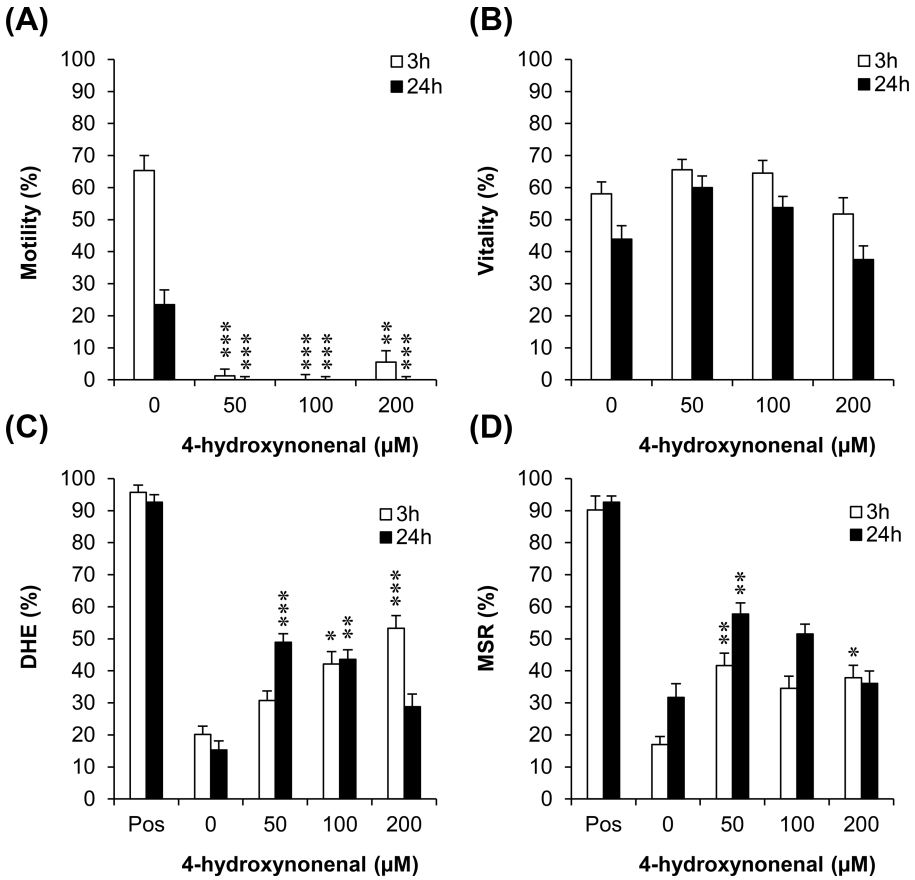


Figure 2. **A**) Motility, **B**) vitality, **C**) total cellular ROS, and **D**) mitochondrial ROS generation following a dose- and time-dependent exposure to 4HNE at 37°C. Positive control (Pos) generated by exposing the same populations of spermatozoa to arachidonic acid (50 μM). Data correspond to mean values ± SEM; n = 9 independent ejaculates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences compared with untreated control sample.

for 15 min at 4°C. The supernatant containing the soluble lysate was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) on a SPECTROstar nano (BMG Labtech, Ortenberg, Germany) and stored at –20°C for up to 1 month until required for western blotting. Protein extracted from spermatozoa was denatured with sodium dodecyl sulfate (SDS) loading buffer (0.2% SDS, 50% 0.375 M Tris, 10% sucrose, 4% β-mercaptoethanol, and 0.001% bromophenol blue) for 10 min at 100°C, prior to being loaded onto a 4%–20% gradient SDS precast polyacrylamide gel (SDS-PAGE; NuSep, Homebush, NSW) and separated by electrophoresis at 110 V for 1.5 h. Proteins were then transferred onto 0.45 μM nitrocellulose membrane (Thermo Fisher Scientific) using standard western blot transfer techniques [45]. Nitrocellulose membranes were blocked in 5% skim milk in Tris buffered saline (TBS; 0.02 M Tris and 0.15 M NaCl) containing 0.1% Tween (TBST) at RT for 1 h prior to incubation with the appropriate antibody; anti-ACR, anti-4HNE, and anti-MDA (1:500) in 1% skim milk in TBST. Immunoblots remained in primary antibody solution overnight at 4°C and were washed three times in TBST before 1 h incubation in goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (HRP; 1:1000 in 1% skim milk in TBST). Nitrocellulose membranes were developed using an enhanced chemiluminescence kit (GE Healthcare, Silverwater, NSW) according to the manufacturer's instructions. Blots were stripped and probed with secondary HRP only to ensure blots were clean prior to commencing a new immunoblotting protocol.

Mass spectrometry

To identify the proteins labeled via immunoblotting, an LC-MS technique was used. Briefly, peptides generated through tryptic digestion [46] were separated by reversed-phase nano-LC (Dionex Ultimate 3000 RSLCnano, Idstein, Germany) before being sequenced by LC–MS/MS on an electrospray ionization 3D ion trap mass spectrometer (AmaZon ETD, Bruker Daltonik, Bremen, Germany). The raw MS/MS files were converted into MASCOT generic format and imported into Bruker Proteinscape platform for database searching. Searches were performed using in-house licensed MASCOT server (version 2.3.02, Matrix Science, London, UK), against the SwissProt database (mammalian taxonomy) and UniprotKB (*Equus caballus*). Trypsin was selected as the digestion enzyme with up to two missed cleavages permitted. Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. Peptide mass tolerances were set at 1.4 and 0.7 Da for parent and daughter fragment ions, respectively. Peptide thresholds were set requiring a false-positive rate 0.05% and a MASCOT score greater than 40. Those spectra meeting these criteria were validated by manual inspection to ensure accurate y- and b-ion detection with overlapping sequence coverage.

Immunoprecipitation

Immunoprecipitation was performed to identify proteins that were covalently modified following electrophilic aldehyde exposure, as

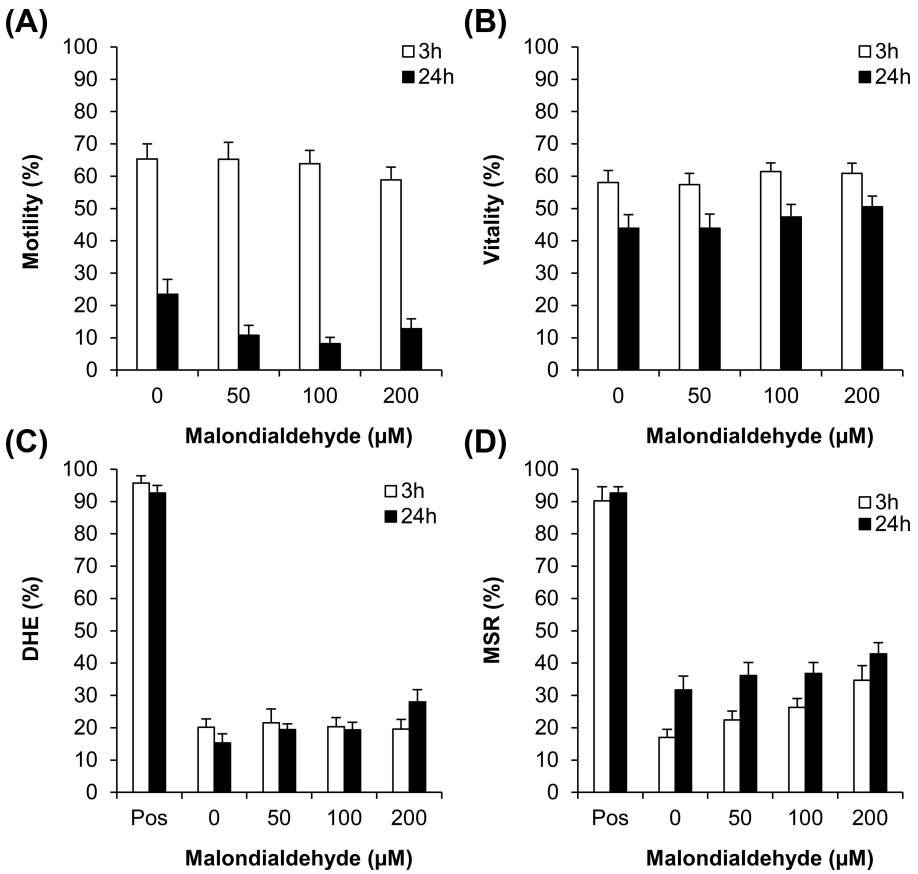


Figure 3. **A**) Motility, **B**) vitality, **C**) total cellular ROS, and **D**) mitochondrial ROS generation following a dose- and time-dependent exposure to MDA at 37°C. Positive control (Pos) generated by exposing the same populations of spermatozoa to arachidonic acid (50 μM). Data correspond to mean values ± SEM; n = 9 independent ejaculates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences compared with untreated control sample.

described previously [47,48]. Cell lysis was performed on 100 × 10^6 cells from each treatment at 4°C for 2 h in IP lysis buffer consisting of 10 mM CHAPS, 10 mM HEPES, 137 mM NaCl, and 10% glycerol with protease inhibitors (Roche). Cells were centrifuged at 14,000 × *g* at 4°C for 20 min, and the recovered supernatant was added to 50 μl aliquots of washed protein G-conjugated Dynabeads (Thermo Fisher Scientific) to preclear at 4°C for 1 h under constant rotation. Meanwhile, 10 μg of target antibody was added to protein G-conjugated Dynabeads and incubated at 4°C for 2 h under constant rotation before undergoing cross-linking using 2 mM 3,3-dithiobis-sulfosuccinimidyl propionate (Thermo Fisher Scientific) as per the manufacturer's instructions. The antibody-bound protein G-conjugated Dynabeads were then resuspended in the total volume of precleared lysate, and co-incubated overnight at 4°C under constant rotation. Following this, antibody–antigen-bound beads were washed three times in PBS before being resuspended in SDS loading buffer and incubated at 100°C for 5 min to elute proteins. The eluted protein was loaded onto a 4%–20% gradient SDS-PAGE gel along with antibody only, bead only, and precleared controls.

Heterologous zona binding assay

The heterologous zona binding assay was performed as described previously [30–32]. Briefly, bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30°C in PBS. Ovaries were washed and the contents of all the follicles aspirated using a 20-gauge needle and 10 ml syringe. Oocytes were allowed to settle in solution and were then manually selected under a dissection microscope, washed, and placed into fresh PBS containing 0.03% hyaluronidase. Cumulus cells were gently removed with a 135 mm denuding pipette (Hunter Scientific, Essex, UK), and zona-intact oocytes were transferred to a high-salt storage solution containing 0.75 M $MgCl_2(H_2O)_6$, 0.5 M $(NH_4)2SO_4$, 40 mM HEPES buffer, and 0.1 mg/ml PVA, and stored at 4°C until use.

Salt-stored oocytes were washed three times in PBS and eight oocytes per treatment were placed in droplets of BWW under water-saturated mineral oil at 37°C in an atmosphere of 5% CO_2, 95% air, and allowed to equilibrate for 30 min. Stallion sperm were collected in noncapacitating (NC) BWW medium (91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin, and 20 mM HEPES buffer with an osmolarity of 300 mOsm/kg) and prepared in the same manner as described above. Spermatozoa were exposed to concentrations of ACR or 4HNE that did not significantly impact motility (5 or 10 μM, respectively) for 3 h at 37°C, and then washed in NC BWW (3 min at 500 × *g*). Spermatozoa were capacitated for 4 h in BWW medium supplemented with 3 mM pentoxifylline, 5 mM dibutyryl cyclic adenosine monophosphate, and 0.5 mM methyl-β-cyclodextrin at a concentration of 10 × 10^6 cells/ml [30]. Cells were incubated at 37°C under an atmosphere of 5% CO_2, 95% air. Noncapacitated cells were incubated for the same period of time in NC BWW. A 2 μl sample of the sperm suspension was added to each droplet of oocytes and co-incubated under the

same conditions for a further 30 min. Oocytes were then washed four times by serial aspiration through droplets of BWW to remove any unbound sperm. Oocytes were mounted on glass slides under coverslips suspended at each corner by 80% paraffin wax and 20% Vaseline gel. The number of motile spermatozoa remaining bound to each zona pellucida was counted by examining the oocytes under phase contrast microscopy, and the data are presented as a percentage of the number of spermatozoa bound in the positive control (i.e., capacitated sperm sample).

Statistical analyses

All experiments were replicated at least three times on spermatozoa purified from independent ejaculates from each of the three pony stallions. Prior to analyses, data were checked for normal distribution using the Shapiro–Wilk test for normality in JMP (SAS Institute Inc., NC, USA, version 11.2.0.). Datasets were not normally distributed, and were consequently analyzed with the nonparametric Wilcoxon test before Dunn with control for joint ranks analysis. Differences with $P < 0.05$ were considered significant. Where n = 9 ejaculates, three semen samples were collected on different days from each of three pony stallions, and in these cases, "stallion ID" was used as a blocking term in the model (random variable).

Results

Electrophilic aldehyde exposure causes motility loss and increases the production of reactive oxygen species in stallion spermatozoa

There was a significant effect of aldehyde treatment on sperm motility ($P < 0.0001$), with no interaction between dose and time observed for ACR or 4HNE treatments. Stallion spermatozoa incubated with ACR suffered a rapid and complete loss of motility that was evident at the shortest time (3 h) and lowest dose (50 μM) assessed compared with the control ($P < 0.0001$; Figure 1A). Motility loss was not associated with a commensurate loss of vitality, which remained consistently high throughout all incubation periods irrespective of ACR concentration (Figure 1B). However, motility loss was associated with significant increases in both total cellular ($P < 0.0001$; Figure 1C) and mitochondrial ROS production ($P < 0.0001$; Figure 1D) compared with the control, which was dose dependent but not time dependent. Thereafter, total cellular and mitochondrial ROS levels appeared to plateau and proved insensitive to subsequent increases in either the concentration of ACR or the duration of exposure to this electrophile.

Similarly, stallion spermatozoa exposed to 4HNE exhibited almost a complete loss of motility after 3 h of treatment compared with the control ($P < 0.0001$; Figure 2A) without any loss of sperm vitality (Figure 2B). 4HNE treatment also significantly increased total cellular (Figure 2C) and mitochondrial ROS generation (Figure 2D) compared with the controls, a result that was dose dependent ($P < 0.0001$), but not time dependent.

There was no impact on sperm motility following MDA exposure, compared with the control (Figure 3A). MDA failed to elicit any significant effect on sperm vitality (Figure 3B), total cellular ROS (Figure 3C), or mitochondrial ROS (Figure 3D), even after 24 h of exposure at the highest concentration.

Accumulation of the electrophilic aldehydes acrolein and 4-hydroxynonenal increases lipid peroxidation

Lipid peroxidation was significantly increased after treatment with ACR compared with the control ($P < 0.0001$; Figure 4A) which

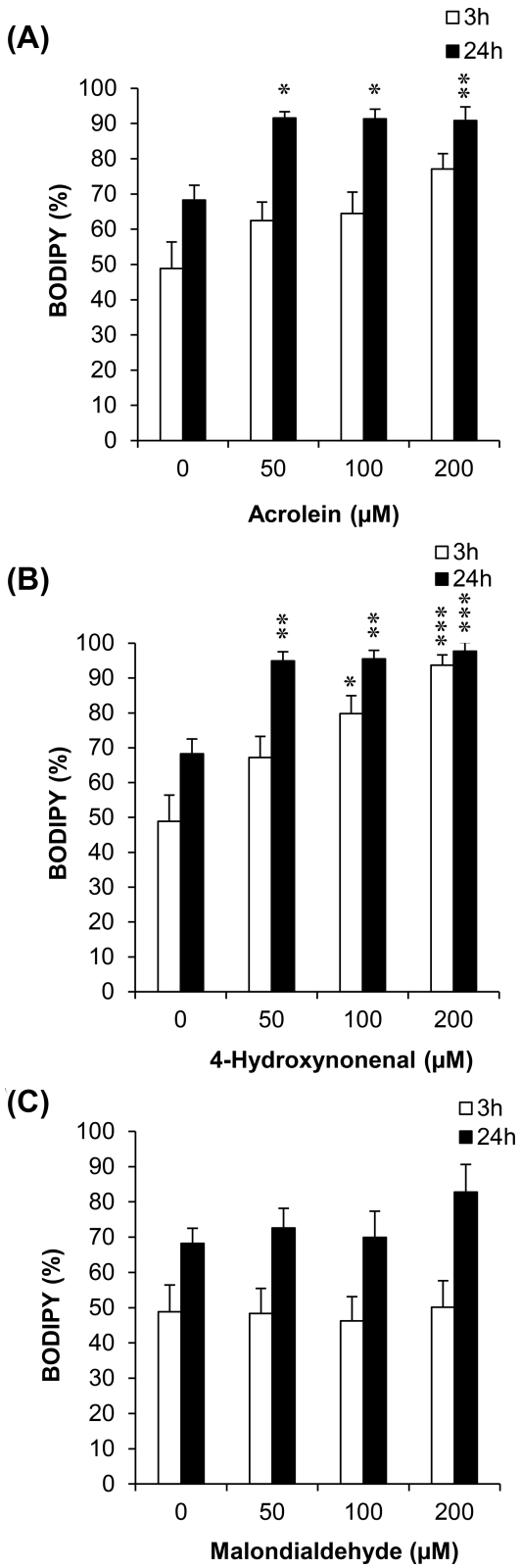


Figure 4. BODIPY positive spermatozoa following a dose- and time-dependent exposure to **A**) ACR, **B**) 4HNE, and **C**) MDA at 37°C. Results expressed as the percentage of live, BODIPY C11 positive cells. Data shown as mean ± SEM; n = 9 independent ejaculates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences compared with untreated control sample.

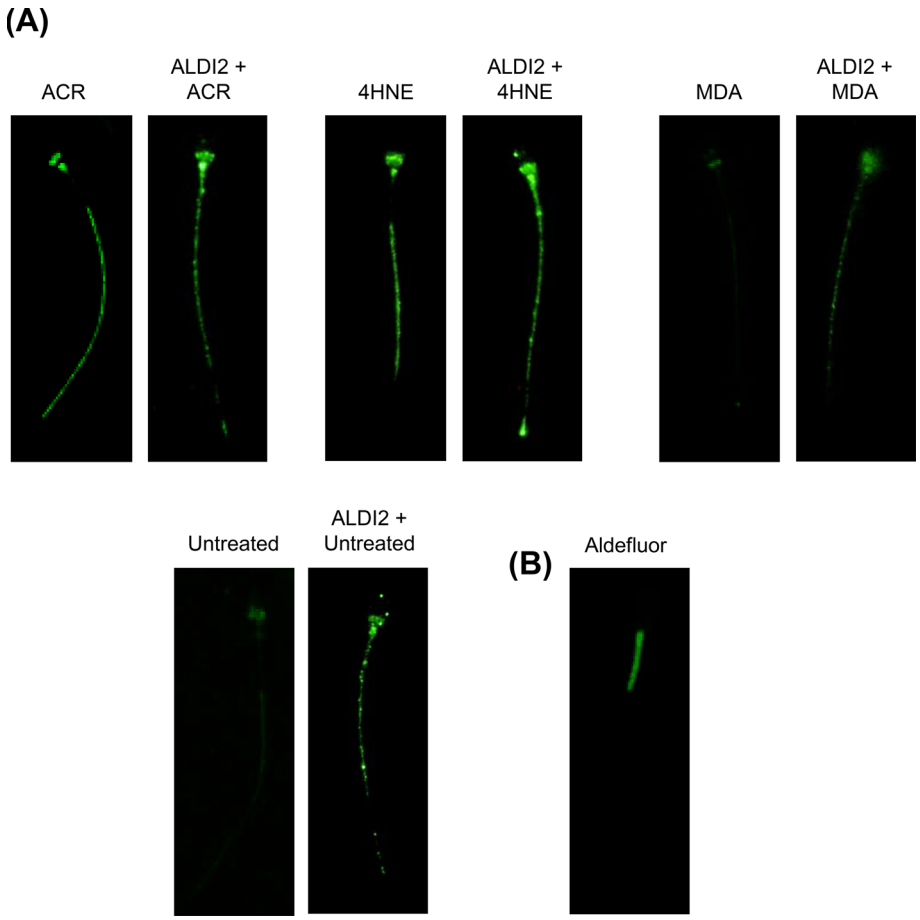


Figure 5. A) Immunolabeling of live spermatozoa following exposure to 50 μM ACR, 4HNE and MDA at 37°C for 3 h. Treatments conducted both in the presence and absence of ALDI2, a potent inhibitor of the ALDH2 enzyme that has been implicated in protection of stallion spermatozoa from oxidative insult. Appropriate anti-aldehyde antibodies show localization of aldehyde adduction (Alexa Fluor 488; green). **B**) Localization of ALDH2 activity (Aldefluor; green) following exposure to 50 μM ACR at 37°C for 3 h. Images are representative of independent ejaculates (n = 3).

was time dependent but not dose dependent. In comparison, 4HNE treatment resulted in an increased BODIPY C11 signal at a lower dose ($P < 0.0001$) and earlier time point ($P < 0.0001$; Figure 4B). No significant increase in lipid peroxidation was detected in spermatozoa treated with MDA at either time point examined, irrespective of dose (Figure 4C).

Electrophilic aldehydes are differentially accumulated in stallion spermatozoa

Immunolabeling of live stallion spermatozoa with anti-ACR, anti-4HNE, and anti-MDA antibodies revealed that each of these aldehydes are differentially adducted to stallion spermatozoa following 3 h exposure (50 μM; Figure 5A). The most pronounced labeling patterns were observed for ACR and 4HNE, both of which were detected within the postacrosomal region of the head, proximal centriole, and principal piece of the sperm flagellum. Notably, neither aldehyde was detected in the anterior region of the sperm head or within the midpiece. The reason for this lack of aldehyde adduction was further investigated by examining the distribution of ALDH (Figure 5B). Treatment with ALDI2 led to a marked accumulation of both ACR and 4HNE immunolabeling within the sperm midpiece, suggesting that the ALDH2 (the ALDH isoform that is localized to the midpiece) [29] activity may, at least in part, account for the protection normally afforded to this critical sperm domain. In contrast to ACR and 4HNE, only relatively modest levels of MDA were able to be detected in stallion spermatozoa following their exposure to this aldehyde. In this regard, weak anti-MDA labeling was observed in the postacrosomal region; however, since both the intensity and pattern of localization resembled that of the secondary antibody only control, this labeling was deemed to be largely nonspecific.

Changes to immunolabeling between time points were also observed in fixed and permeabilized ACR- and 4HNE-treated sperm (Figure 6). In comparison to the 3 h time point where no midpiece staining was evident, by 24 h anti-ACR and anti-4HNE antibodies could be visualized at this site, suggesting ALDH2 is exacerbated following aldehydes exposure. Notably, the fixation and permeabilization of these cells also facilitated the identification of an additional site of both ACR and 4HNE adduction located within the anterior region of the sperm head.

Heat shock protein 90AA1 and arylsulfatase A are targets for acrolein and 4-hydroxynonenal electrophilic aldehyde adduction

Electrophilic aldehyde-treated sperm lysates were subjected to immunoblotting with the corresponding anti-aldehyde antibody (Figure 7A–C). This analysis revealed ACR adduction to a predominant

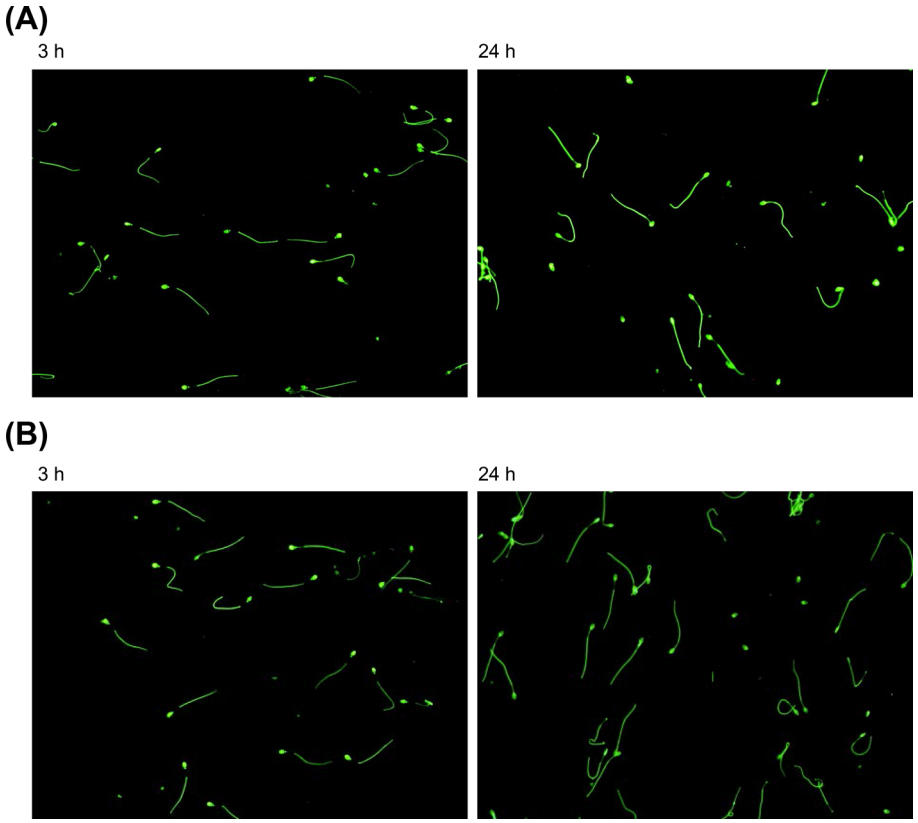


Figure 6. Immunolabeling of fixed and permeabilized spermatozoa following exposure to 50 μM **A**) ACR, and **B**) 4HNE at 37°C for 3 and 24 h. Appropriate anti-aldehyde antibodies show localization of aldehyde adduction (Alexa Fluor 488; green). Images are representative of independent ejaculates (n = 3).

protein band of 90 kDa in addition to a number of less intensely labeled bands ranging in molecular weight from ~30 to 120 kDa. A similar 90 kDa band was also a substrate for 4HNE adduction, although this aldehyde appeared to bind more prominently to a 60 kDa protein. In marked contrast, MDA treatment, which was previously shown to induce negligible lipid peroxidation and ROS generation, also failed to promote the appreciable adduction of any sperm proteins. This finding was also consistent with MDA immunolabeling (Figure 5A).

Mass spectrometry led to high confidence identification of HSP90AA1 and ARSA as potential targets for adduction by ACR or 4HNE. Two strategies were subsequently undertaken to confirm the vulnerability of these proteins as targets for adduction.

In the first approach, both HSP90AA1 and ARSA were colocalized with ACR and 4HNE, respectively. In fixed and permeabilized spermatozoa, HSP90AA1 was strongly labeled within the postacrosomal region of the head, midpiece, and principle piece of the sperm tail (Figure 7D). In comparison, ARSA was localized throughout the entire sperm head, proximal centriole, midpiece, and tail (Figure 7E). In each instance, protein labeling colocalized to regions in which ACR and 4HNE preferentially accumulate. We next conducted reciprocal pull downs to confirm aldehyde adduction of these proteins in treated spermatozoa (Figure 8A and B). These data confirm that HSP90AA1 and ARSA are indeed targeted for adduction by electrophilic aldehydes in stallion spermatozoa experiencing oxidative stress.

Zona pellucida binding competence is decreased after treatment with low dose acrolein and 4-hydroxynonenal

Spermatozoa were treated with low levels of ACR and 4HNE to determine a dose at which motility was not significantly impacted (Figure 9A and B). These doses were then used to examine the impact of ACR and 4HNE on zona pellucida binding competence. A significantly higher number of spermatozoa was able to bind to the zona pellucida following capacitation compared with spermatozoa incubated under NC conditions (13.77 ± 1.56% of the control; $P < 0.0001$; Figure 9C). Despite comparable levels of motility between untreated and treated samples, spermatozoa treated with low levels of ACR and 4HNE prior to capacitation had significantly lower zona binding competence compared with the capacitated control (10.53 ± 1.61% and 5.75 ± 2.39% of the control, respectively; $P < 0.0001$).

Discussion

Because of a dependence on OXPHOS for ATP production [16] and an abundance of PUFAs in their plasma membrane [4,7,49], stallion spermatozoa appear to have coopted detoxification strategies that enable enhanced resistance to oxidative stress compared with the spermatozoa of other well-studied mammalian species. Notwithstanding such adaptations, this study has revealed that stallion spermatozoa remain particularly susceptible to oxidative adducts that are

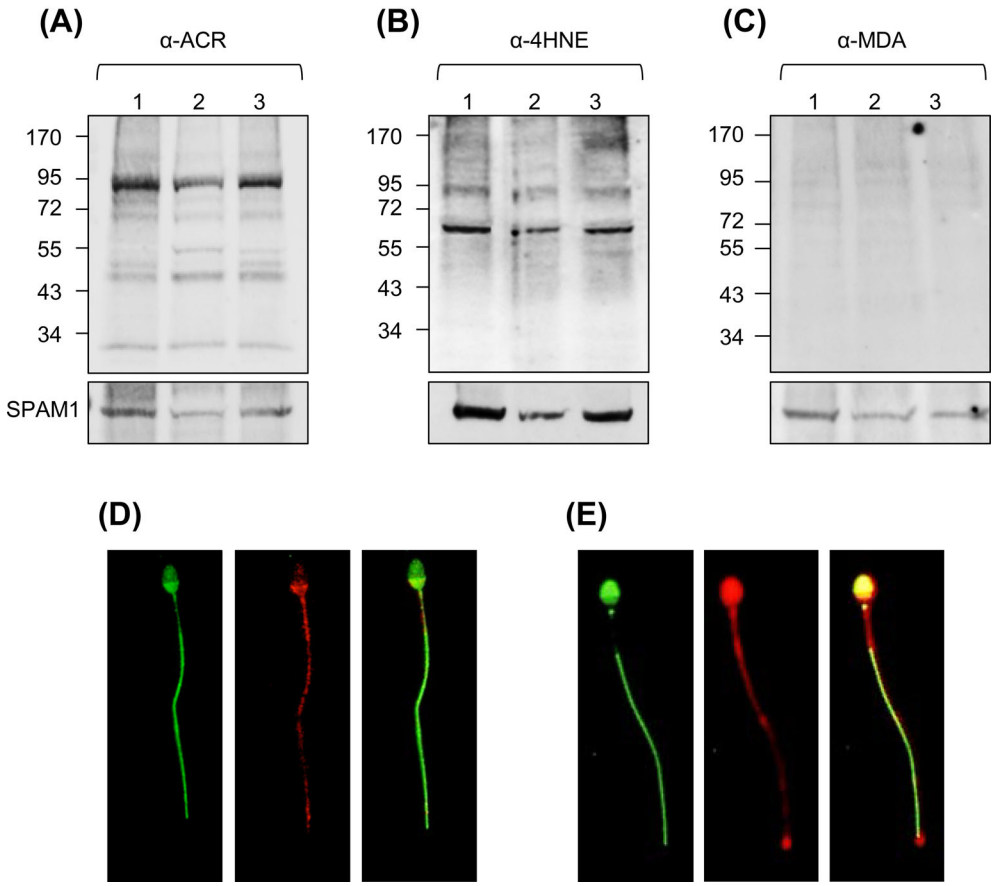


Figure 7. Western blot analysis of sperm protein lysates following exposure to 50 μM **A**) ACR, **B**) 4HNE, and **C**) MDA at 37°C for 3 h. Each lane represents a different stallion. Loading control SPAM1. Fixed and permeabilized spermatozoa colocalized with **D**) ACR (Alexa Fluor 488; green) and HSP90AA1 (Alexa Fluor 594; red), and **E**) 4HNE (Alexa Fluor 488; green) and ARSA (Alexa Fluor 594; red) following 50 μM aldehyde exposure at 37°C for 3 h. Images are representative of all replicates (n = 3).

formed upon exposure of the cells to intermediate products of the oxidative stress cascade, such as ACR and 4HNE. Thus, ACR and 4HNE exposure elicited a higher degree of acute motility loss, superoxide production, and lipid peroxidation than previously reported following equivalent treatment of human spermatozoa [6,41,43]. In this study, motility loss and ROS generation preceded lipid peroxidation, which may reflect the disruption of mitochondrial function accompanied by electron leakage to oxygen, a phenomenon that has been previously reported following exposure to reactive aldehydes [7,48,49]. Despite the different rates at which spermatozoa succumb to oxidative insult, the impact of ACR and 4HNE exposure on stallion and human sperm function is remarkably similar. Such findings serve to reinforce the unique vulnerability of the male germ cell to oxidative attack resulting from the adduction of key proteins, including the antioxidants glutathione [50] and ALDH [51].

A clear difference exists between the localization of aldehyde adducts in horse and human spermatozoa [41,43], suggesting possible differences between these two species in the nature of the target proteins. The proximal centriole was a target for adduction by ACR and 4HNE, likely due to its positive charge. Importantly, the proximal centriole regulates cell division and adduction can result in abnormal cleavage and fertilization failure [52]. In human spermatozoa, electrophilic aldehydes bind primarily to the mitochondria and the major protein adducted by 4HNE was shown to be of mitochondrial origin (succinic acid dehydrogenase) [7,41]. In contrast, adducts were notably absent from the mitochondria of stallion spermatozoa following 3 h aldehyde treatment. Such differences may, however, simply be a function of the duration of exposure. In the short term, ALDH2 in the mitochondria protects these organelles from aldehyde attack. Thus, ALDH2 is localized to the mitochondria of the sperm midpiece, while the exclusion of ACR and 4HNE binding to this region of the cell suggests that this enzyme is able to metabolize electrophilic aldehydes to their respective carboxylic acids before they have the opportunity to form adducts with proteins essential for normal cellular function (Figure 10). However, it is likely this mechanism is short lived because the aldehydes metabolized by this enzyme can form a Michael adduct or Schiff base with a key cysteine at the catalytic site of the enzyme [29,51]. Once ALDH2 activity has been exhausted, vulnerable proteins, particularly those in the mitochondria themselves, can subsequently be adducted, demonstrated by intense aldehyde staining patterns throughout the cell, including the midpiece region [29]. This conclusion was reinforced by treating spermatozoa with the ALDH inhibitor, ALDI2, which led to mitochondrial aldehyde adduction in the absence of ALDH activity (Figure 5). This subcellular staining was mirrored after 24 h incubation with both ACR and 4HNE (in the absence of ALDI2 treatment), indicating that after 24 h incubation with these electrophiles, ALDH2 activity is lost (Figure 6).

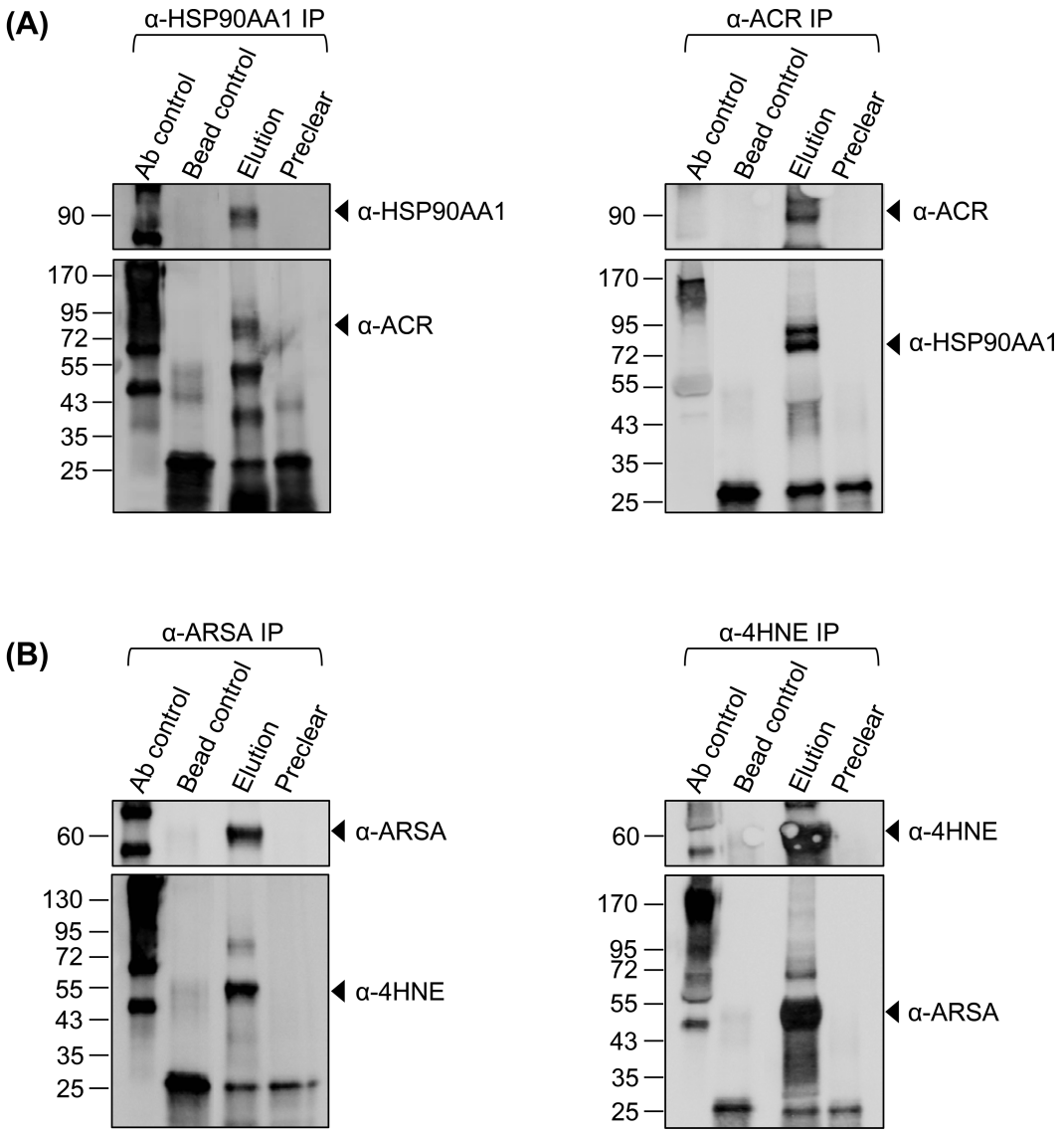


Figure 8. Reciprocal pull-downs and IP with **A**) HSP90AA1 and ACR, and **B**) ARSA and 4HNE following 50 μM aldehyde exposure at 37°C for 3 h. Images are representative of all replicates (n = 3).

While ALDH2 appears to play a vital role in the detoxification of aldehydes in stallion spermatozoa, other enzymes, particularly glutathione-S-transferases and glutathione peroxidase [18,53,54], provide additional aldehyde detoxification roles [49,55,56]. In addition to binding aldehydes through cysteine residues and redirecting them out of the cell, glutathione-S-transferases protect protein thiol groups which are known to be essential for sperm survival [49,56–58]. Indeed, a recent study in stallion spermatozoa revealed that both ALDH2 and glutathione-S-transferase activity reduced levels of ROS produced by the mitochondria. Nonetheless, ALDH2 activity alone was of greater benefit than glutathione [29]. Interestingly, ALDH2 also plays an important role in spermatogenesis, during retinoic acid biosynthesis. In a study by Amory [59], infertile men exhibited lower levels of ALDH2 in testicular tissue, reinforcing the importance of ALDH2 in sperm metabolism, and opening up new possibilities for contraceptive development.

Aside from ALDH2, HSP90AA1 and ARSA were also identified as substrates for ACR and 4HNE adduction, through the ability of these powerful electrophiles to covalently modify cysteine, lysine, or histidine residues on key proteins [60]. HSP90 has been investigated for its role in human infertility [61] and has previously been shown to play a crucial role in the motility of boar sperm [48]. In a study by Carbone et al. [62], HSP90 was modified and inactivated by reactive aldehydes. In comparison, ARSA is a zona pellucida receptor molecule expressed on the surface of spermatozoa [63] which has also been hypothesized to be an important mediator in sperm-egg recognition in horses [64]. Both proteins are essential for cellular function, and consequently have the capacity to impede normal sperm function when targeted by lipid aldehydes [41,43]. In this study, low levels of ACR and 4HNE were sufficient in significantly decreasing zona binding competence (Figure 9), reinforcing the aptitude of electrophilic aldehydes for targeting proteins with important function [38].

There is a paucity of literature characterizing sperm proteins spontaneously adducted by ACR; however, proteins covalently modified by 4HNE have been widely examined as the aldehyde has

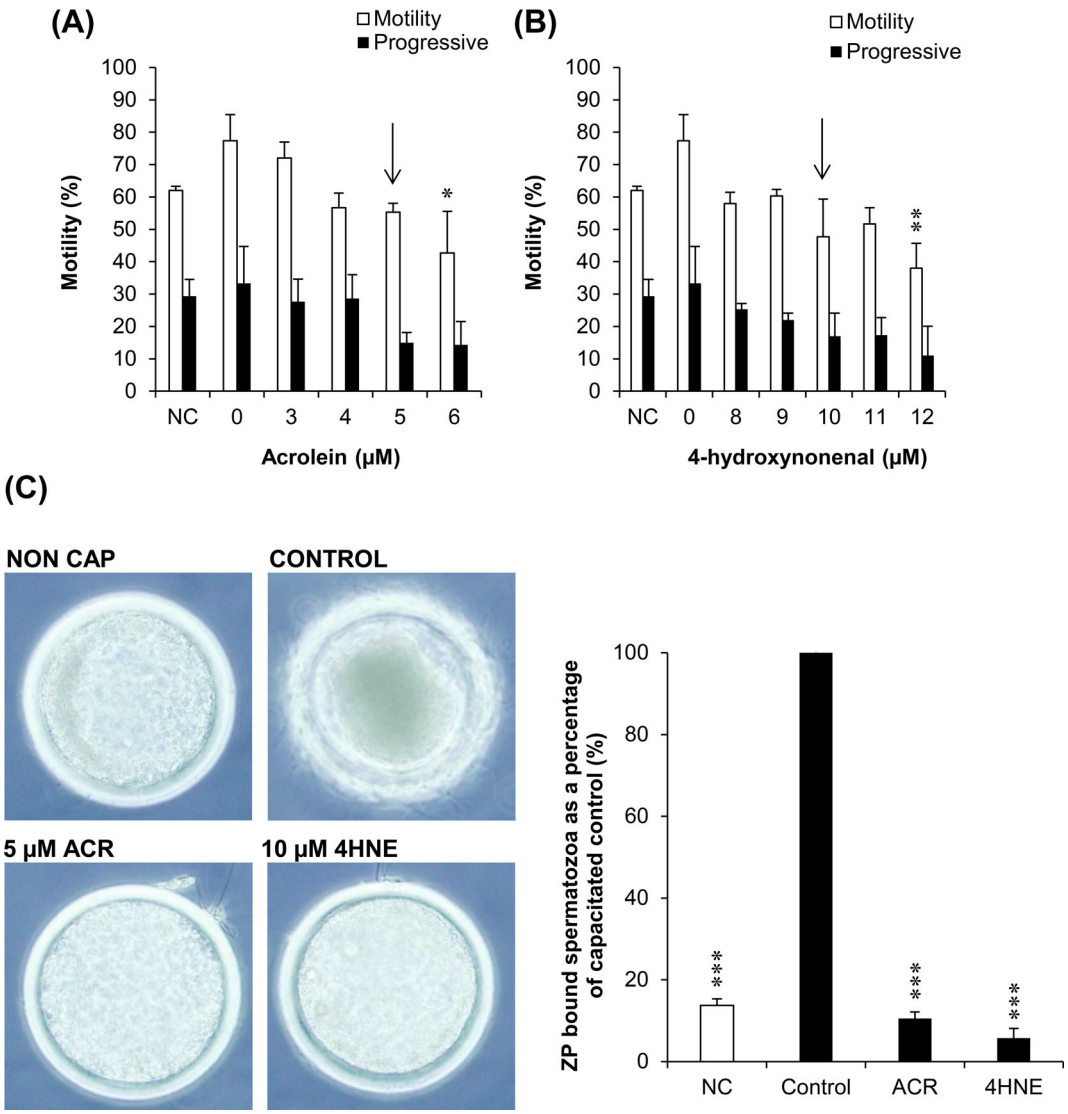


Figure 9. Total and progressive motility following a low dose-dependent exposure to **A**) ACR and **B**) 4HNE at 37°C for 3 h. **C**) Zona binding competence following exposure to 5 μM ACR and 10 μM 4HNE. Data correspond to mean values ± SEM; n = 3 independent ejaculates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for differences compared with untreated control sample.

proven cytotoxicity [41] and is the most abundant aldehyde produced following lipoperoxidative damage [38,65,66]. The modification of mitochondrial electron transport chain proteins by 4HNE has been well described, a phenomenon which results in electron leakage and superoxide production by the mitochondria [6,43], along with irreversible damage to the molecular chaperones essential for sperm-egg recognition [67]. Target substrates in human spermatozoa include succinic acid dehydrogenase [43], HSP70 [43], HSPA2 [67], and dynein heavy chain [68]. Consistent with our findings in stallion spermatozoa, 4HNE adduction of HSPA2 in human spermatozoa led to the perturbation of ARSA expression due to its role within the HSPA2/ARSA/SPAM1 protein complex [67]. As regulation of this protein complex is necessary for sperm-egg recognition, modification of such an important protein renders the cell incapable of fertilization. Although the degree of similarity that exists between the protein complexes in human and stallion spermatozoa is not known, our findings suggest that lipid aldehydes might trigger similar pathophysiological mechanisms across a range of species.

Historically, MDA has played an important role as a marker for oxidative stress [15,38,69–72], as it is a product of lipid peroxidation under normal physiological conditions. However, in this study, there was no evidence of ROS generation, lipid peroxidation, or protein adduction following treatment with MDA. This follows on from previous findings reporting MDA as less reactive than 4HNE and ACR [30,32]. Indeed, exposure to MDA showed no pertinent increases in ROS or lipid peroxidation of human spermatozoa and as such was determined to be relatively noncytotoxic [41]. These findings suggest that while MDA is a suitable biomarker of lipoperoxidative damage, it makes little direct contribution to the loss of sperm function observed following the induction of oxidative stress.

Initial findings suggested that the impact of ACR and 4HNE exposure had similar impacts on stallion spermatozoa; however, when

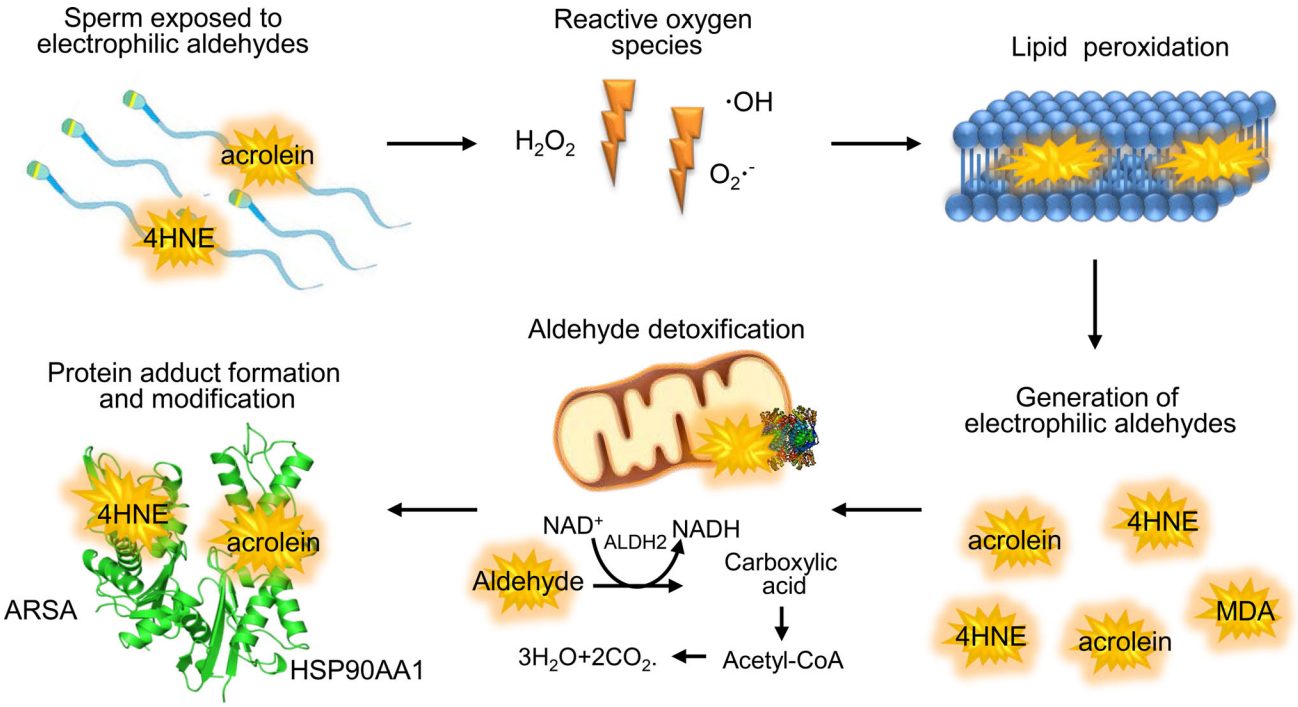


Figure 10. Accumulation of electrophilic aldehydes and the role of ALDH2 in the oxidative stress pathway. Normal stallion sperm metabolism (OXPHOS) results in the formation of ROS, leading to covalent lipid adduct formation and peroxidation, and the generation of electrophilic aldehydes. This process is exacerbated when stallion spermatozoa are incubated with aldehydes such as ACR and 4HNE. ALDH2 facilitates the removal of aldehydes from the mitochondria as they accumulate, via a step-by-step detoxification process; however, this mechanism is short lived. ACR and 4HNE bind to the cysteine residues of HSP90AA1 and ARSA, respectively, forming protein adducts. HSP90AA1 and ARSA proteins are covalently modified.

investigated at lower doses, ACR treatment led to a significant decrease in zona binding competence at half the dose of 4HNE, suggesting a higher level of cytotoxicity. This is supported by previous studies [41,50] regarding ACR as the strongest electrophile with the highest reactivity to proteins. Studies by Moazamian et al. [41] in human sperm reported that reactivity to glutathione by ACR was a log order more reactive than 4HNE. A varied response of 4HNE production exists during oxidative insult, estimated between 10 μM and 5 mM [38], whereas ACR production is estimated to occur between 30 and 180 μM [37,39,40] and is able to exert similar impacts. It would be useful to investigate the abundance of ACR in comparison to 4HNE under normal physiological conditions in stallion spermatozoa to allow further insight into the oxidative stress challenge. A secondary explanation for stallion spermatozoa's sensitivity to low levels of electrophilic aldehydes may be the lack of environmental exposure to toxins such as cigarette smoke and pesticides compared with humans.

A delicate balance clearly exists between ROS produced during normal OXPHOS metabolism and downstream production of electrophilic aldehydes which impair protein function and contribute to an oxidative stress cascade [2]. In this study, the exposure of stallion spermatozoa to the electrophilic aldehydes ACR and 4HNE induced protein adducts, leading to increased ROS generation, increased lipid peroxidation, and a loss of motility. Conversely, MDA exposure had little impact on cell function. Aldehyde localization revealed that ALDH2 may be responsible for acutely rescuing mitochondrial proteins susceptible to adduction by ACR and 4HNE; however, ALDH2 activity is short lived when cells are subjected to extended aldehyde exposure. Our findings also reveal the disruption of functional sperm proteins, HSP90AA1 and ARSA, and a decrease in zona binding competence. Future work will focus on refining our analysis of the functional implications of electrophilic aldehydes and oxidative stress on stallion spermatozoa and determining whether this information can be exploited for the development of diagnostic markers of stallion fertility.

Acknowledgment

The authors thank Sarah Lambourne and Aleona Swegen for assistance in stallion collection.

REFERENCES

1. Halliwell B, Gutteridge JM. *Free Radicals in Biology and Medicine*. Oxford, UK: Oxford University Press; 2003:199.
2. Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* 2011; **14**:367–381.
3. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008; **93**:3199–3207.
4. Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* 2010; **48**:112–119.
5. Du Plessis S, McAllister D, Luu A, Savia J, Agarwal A, Lampiao F. Effects of H2O2 exposure on human sperm motility parameters, reactive oxygen species levels and nitric oxide levels. *Andrologia* 2010; **42**:206–210.

6. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol Reprod* 2012; **87**:110.
7. Aitken RJ, Baker MA. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. *Int J Dev Biol* 2013; **57**:265–272.
8. Hughes LM, Griffith R, Carey A, Butler T, Donne SW, Beagley KW, Aitken RJ. The spermostatic and microbicidal actions of quinones and maleimides: toward a dual-purpose contraceptive agent. *Mol Pharmacol* 2009; **76**:113–124.
9. Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987; **81**:459–469.
10. Aitken RJ, Wingate JK, De Iuliis GN, Koppers AJ, McLaughlin EA. Cis-unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. *J Clin Endocrinol Metab* 2006; **91**:4154–4163.
11. De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 2009; **81**:517–524.
12. Aitken R, De Iuliis G. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod* 2010; **16**:3–13.
13. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 1979; **31**:531–537.
14. Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod* 2007; **13**:203–211.
15. Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Androl* 1998; **21**:81–94.
16. Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. *Biol Reprod* 2014; **91**:77.
17. Ferrusola CO, Fernandez LG, Sandoval CS, Garcia BM, Martinez HR, Tapia J, Peña F. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. *Theriogenology* 2010; **74**:458–465.
18. Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006; **8**:1865–1879.
19. Ferrusola CO, Fernández LG, Morrell J, Sandoval CS, García BM, Rodríguez-Martinez H, Tapia J, Peña F. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* 2009; **138**:55–63.
20. Ball BA, Gravance CG, Medina V, Baumber J, Liu IK. Catalase activity in equine semen. *Am J Vet Res* 2000; **61**:1026–1030.
21. Vernet P, Aitken R, Drevet J. Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* 2004; **216**:31–39.
22. Hemachand T, Shaha C. Functional role of sperm surface glutathione S-transferases and extracellular glutathione in the haploid spermatozoa under oxidative stress. *FEBS Lett* 2003; **538**:14–18.
23. Kwon W-S, Oh S-A, Kim Y-J, Rahman MS, Park Y-J, Pang M-G. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci Rep* 2015; **5**:13821.
24. Hering D, Lecewicz M, Kordan W, Majewska A, Kaminski S. Missense mutation in glutathione-S-transferase M1 gene is associated with sperm motility and ATP content in frozen-thawed semen of Holstein-Friesian bulls. *Anim Reprod Sci* 2015; **159**:94–97.
25. Roshdy O, Hussein T, Zakaria N, Sabry A. Glutathione S-transferase Mu-1 gene polymorphism in Egyptian patients with idiopathic male infertility. *Andrologia* 2015; **47**:587–593.
26. Mann T. Biochemistry of stallion semen. *J Reprod Fertil Suppl* 1975; **26**:47–52.
27. Choudhary S, Xiao T, Srivastava S, Zhang W, Chan L, Vergara L, Van Kuijk F, Ansari N. Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. *Toxicol Appl Pharmacol* 2005; **204**:122–134.
28. Swegen A, Curry BJ, Gibb Z, Lambourne SR, Smith ND, Aitken RJ. Investigation of the stallion sperm proteome by mass spectrometry. *Reproduction* 2015; **149**:235–244.
29. Gibb Z, Lambourne SR, Curry BJ, Hall SE, Aitken RJ. Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. *Biol Reprod* 2016; **94**:1–11.
30. Bromfield EG, Aitken RJ, Gibb Z, Lambourne SR, Nixon B. Capacitation in the presence of methyl-β-cyclodextrin results in enhanced zona pellucida-binding ability of stallion spermatozoa. *Reproduction* 2014; **147**:153–166.
31. da Silva MAC, Seidel GE, Squires EL, Choi YH, Carnevale EM. Binding of stallion sperm to equine and bovine zonae pellucidae: effect of milk, milk proteins and glucose. *Reprod Fertil Dev* 2004; **16**:252.
32. Clulow JR, Evans G, Maxwell WMC, Morris LHA. Evaluation of the function of fresh and frozen-thawed sex-sorted and non-sorted stallion spermatozoa using a heterologous oocyte binding assay. *Reprod Fertil Dev* 2010; **22**:710–717.
33. Biggers J, Whitten W, Whittingham D. The culture of mouse embryos in vitro. In: Daniels J (ed.), *Methods in Mammalian Embryology*. San Fransisco: Freeman; 1971: 86–116.
34. Aurich C, Spergser J. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology* 2007; **67**:912–918.
35. Kenney R, Bergman R, Cooper W, Morse G. Minimal contamination techniques for breeding mares: technique and preliminary findings. *Proc AAEP* 1975; **21**:327–336.
36. Aitken R, Ryan A, Curry B, Baker M. Multiple forms of redox activity in populations of human spermatozoa. *Mol Hum Reprod* 2003; **9**:645–661.
37. Sakata K, Kashiwagi K, Sharmin S, Ueda S, Irie Y, Murotani N, Igarashi K. Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients. *Biochem Biophys Res Commun* 2003; **305**:143–149.
38. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog Lipid Res* 2003; **42**:318–343.
39. Eiserich JP, van der Vliet A, Handelman GJ, Halliwell B, Cross CE. Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. *Am J Clin Nutr* 1995; **62**:1490s–1500s.
40. Satoh K, Yamada S, Koike Y, Igarashi Y, Toyokuni S, Kumano T, Takahata T, Hayakari M, Tsuchida S, Uchida K. A 1-hour enzyme-linked immunosorbent assay for quantitation of acrolein- and hydroxynonenal-modified proteins by epitope-bound casein matrix method. *Anal Biochem* 1999; **270**:323–328.
41. Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, Aitken RJ. Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod* 2015; **21**:502–515.
42. Aitken R, Smith T, Lord T, Kuczera L, Koppers A, Naumovski N, Connaughton H, Baker M, Iuliis G. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology* 2013; **2**:192–205.
43. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem* 2012; **287**:33048–33060.
44. Khanna M, Chen C-H, Kimble-Hill A, Parajuli B, Perez-Miller S, Baskaran S, Kim J, Dria K, Vasiliou V, Mochly-Rosen D. Discovery of a novel class of covalent inhibitor for aldehyde dehydrogenases. *J Biol Chem* 2011; **286**:43486–43494.
45. Koppers A, Mitchell L, Wang P, Lin M. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated

with motility loss and oxidative DNA damage in human spermatozoa. *Biochem J* 2011; **436**:687–698.
46. Luque-Garcia JL, Neubert TA. On-membrane tryptic digestion of proteins for mass spectrometry analysis. *Methods Mol Biol* 2009; **536**:331–341.
47. Bromfield E, Aitken RJ, Nixon B. Novel characterization of the HSPA2-stabilizing protein BAG6 in human spermatozoa. *Mol Hum Reprod* 2015; **21**:755–769.
48. Lord T, Martin JH, Aitken RJ. Accumulation of electrophilic aldehydes during post-ovulatory ageing of mouse oocytes causes reduced fertility, oxidative stress, and apoptosis. *Biol Reprod* 2015; **92**:33.
49. Perluigi M, Coccia R, Butterfield DA. 4-Hydroxy-2-nonenal, a reactive product of lipid peroxidation, and neurodegenerative diseases: a toxic combination illuminated by redox proteomics studies. *Antioxid Redox Signal* 2012; **17**:1590–1609.
50. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991; **11**:81–128.
51. Doorn JA, Hurley TD, Petersen DR. Inhibition of human mitochondrial aldehyde dehydrogenase by 4-hydroxynon-2-enal and 4-oxonon-2-enal. *Chem Res Toxicol* 2006; **19**:102–110.
52. Schatten H, Sun Q-Y. The role of centrosomes in mammalian fertilization and its significance for ICSI. *Mol Hum Reprod* 2009; **15**:531–538.
53. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. *J Biol Chem* 2008; **283**:21837–21841.
54. Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 1994; **16**:259–267.
55. Drevet JR. The antioxidant glutathione peroxidase family and spermatozoa: a complex story. *Mol Cell Endocrinol* 2006; **250**:70–79.
56. Vander Jagt DL, Hunsaker LA, Vander Jagt TJ, Gomez MS, Gonzales DM, Deck LM, Royer RE. Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. *Biochem Pharmacol* 1997; **53**:1133–1140.
57. Aitken R, Flanagan H, Connaughton H, Whiting S, Hedges A, Baker M. Involvement of homocysteine, homocysteine thiolactone, and paraoxonase type 1 (PON-1) in the etiology of defective human sperm function. *Andrology* 2016; **4**:345–360.
58. Martin Munoz P, Ortega Ferrusola C, Vizuete G, Plaza Davila M, Rodriguez Martinez H, Pena FJ. Depletion of intracellular thiols and increased production of 4-hydroxynonenal that occur during cryopreservation of stallion spermatozoa lead to caspase activation, loss of motility, and cell death. *Biol Reprod* 2015; **93**:143.
59. Amory JK, Arnold S, Lardone MC, Piottante A, Ebensperger M, Isoherranen N, Muller CH, Walsh T, Castro A. Levels of the retinoic acid synthesizing enzyme aldehyde dehydrogenase-1A2 are lower in testicular tissue from men with infertility. *Fertil Steril* 2014; **101**:960–966.
60. Eliuk SM, Renfrow MB, Shonsey EM, Barnes S, Kim H. Active site modifications of the brain isoform of creatine kinase by 4-hydroxy-2-nonenal correlate with reduced enzyme activity: mapping of modified sites by Fourier transform-ion cyclotron resonance mass spectrometry. *Chem Res Toxicol* 2007; **20**:1260–1268.
61. Pires ES, Choudhury AK, Idicula-Thomas S, Khole VV. Anti-HSP90 autoantibodies in sera of infertile women identify a dominant, conserved epitope EP6 (380-389) of HSP90 beta protein. *Reprod Biol Endocrinol* 2011; **9**:16.
62. Carbone DL, Doorn JA, Kiebler Z, Ickes BR, Petersen DR. Modification of heat shock protein 90 by 4-hydroxynonenal in a rat model of chronic alcoholic liver disease. *J Pharmacol Exp Ther* 2005; **315**:8–15.
63. Redgrove KA, Anderson AL, McLaughlin EA, O'Bryan MK, Aitken RJ, Nixon B. Investigation of the mechanisms by which the molecular chaperone HSPA2 regulates the expression of sperm surface receptors involved in human sperm–oocyte recognition. *Mol Hum Reprod* 2013; **19**:120–135.
64. Mugnier S, Boittin S, Douet C, Monget P, Magistrini M, Goudet G. The involvement of beta-1, 4-galactosyltransferase and N-acetylglucosamine residues in fertilization has been lost in the horse. *Reprod Biol Endocrinol* 2008; **6**:51.
65. Benedetti A, Comporti M, Esterbauer H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta* 1980; **620**:281–296.
66. Schauenstein E, Esterbauer H. Formation and properties of reactive aldehydes. In: *Submolecular Biology and Cancer*, vol. 67. Amsterdam: Excerpta Medica; 1979: 225–244.
67. Bromfield EG, Aitken RJ, Anderson AL, McLaughlin EA, Nixon B. The impact of oxidative stress on chaperone-mediated human sperm–egg interaction. *Hum Reprod* 2015; **30**:2597–2613.
68. Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J, Gordon C. Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* 2015; **92**:108.
69. Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 1995; **7**:659–668.
70. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl* 2000; **21**:895–902.
71. Neild D, Brouwers J, Colenbrander B, Agüero A, Gadella B. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. *Mol Reprod Dev* 2005; **72**: 230–238.
72. Aitken R, Harkiss D, Buckingham D. Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev* 1993; **35**: 302–315.

CHAPTER 4

Active immunization with sperm proteins covalently modified by the lipid peroxidation product, acrolein

Authors: Hall SE, Gibb Z, Nixon B, Swegen A, Aitken RJ

Chapter 4: Overview

Following the findings in the previous chapter, it was hypothesised that proteins covalently modified by ACR or 4HNE could lead to an enhanced autoimmune response that would offer a long-lasting contraceptive effect. During the process of aldehyde adduct formation, susceptible proteins undergo covalent modification and become highly immunogenic, thus increasing susceptibility for recognition by the immune system. Indeed, previous studies investigating the onset of immune disease have demonstrated that aldehyde adduct formation on proteins is critical in generating antibodies against self antigens. To assess this hypothesis, a proof-of-concept immunisation trial was performed in the mouse model.

In this chapter, mouse spermatozoa were exposed to electrophilic aldehydes, ACR and 4HNE, and surface proteins were extracted and formulated into an immunisation suspension with an equal volume of adjuvant. A preliminary immunisation trial was conducted in both male and female mice prior to mating to assess reproductive outcomes; however, the focus of this study was in the male.

Our attention was immediately directed to mice immunised with ACR-adducted sperm proteins as there was a decrease in the number of offspring in the mated cohort. To explain the reduction in fertility and zona pellucida binding competence, immunoblotting was performed on sperm lysates of ACR groups both *in vivo* and *in vitro*. A predominant band at 60 kDa was confirmed via mass spectrometry and immunoprecipitation to contain four potential protein targets each of which have been implicated in gamete interaction, including: heat shock protein 60 (HSP60) and arylsulfatase A (ARSA), both implicated in mediation of sperm-zona pellucida recognition/adhesion, and izumo sperm-egg fusion 1 (IZUMO1) and protein disulfide isomerase A6 (PDIA6), both linked to sperm-egg fusion. Since these proteins each hold putatively important roles in fertilisation, their modification may at least, in part,

account for the notable decrease in sperm-zona pellucida binding competence and reduced number of foetuses observed following immunisation with a cocktail of aldehyde-modified sperm proteins. This study thus contributes valuable findings pertinent for the development of a lifelong immunocontraceptive for domestic and pest animals.

Active immunization with sperm proteins covalently modified by the lipid peroxidation product, acrolein[†]

Running title: Immunization with covalently modified sperm proteins

Sally E. Hall[1,2]*, Zamira Gibb[1], Brett Nixon[1], Aleona Swegen[1,2] and R. John Aitken[1,2]

[1]Priority Research Centre for Reproductive Science, Discipline of Biological Sciences, Faculty of Science and Information Technology, University of Newcastle, Callaghan, New South Wales, Australia and [2]Invasive Animals Cooperative Research Centre, University of Canberra, Bruce, Australian Capital Territory, Australia.

*Correspondence: Priority Research Centre for Reproductive Science, Level 4, Life Sciences Building, Ring Road, Discipline of Biological Sciences, Faculty of Science and Information Technology, University of Newcastle, Callaghan, NSW, 2308, Australia. Email: sally.e.hall@uon.edu.au

[†]Supported by Invasive Animals Cooperative Research Centre (#2.C.13).

Presented in part at the 47th annual meeting of the Society for Reproductive Biology, 21-24 August 2016, Gold Coast, Queensland.

Abstract

The development of a safe, effective, species-specific immunocontraceptive capable of inducing prolonged suppression of fertility in domestic and feral animals is currently hindered by the lack of an appropriate target antigen, particularly in the male. In order to address this issue, we have explored the hypothesis that the auto- and iso- antigenicity of sperm proteins could be enhanced by the formation of adducts with electrophilic lipid aldehydes generated as a consequence of lipid peroxidation. In the course of this study, mice were immunized with sperm lysates in their native state or following reaction with the electrophilic aldehydes, acrolein or 4-hydroxynonenal (4HNE). Only mice immunized with acrolein-adducted sperm proteins mounted an immune response sufficient to reduce fertility resulting in an approximate halving of litter size (acrolein: 4.66 ± 4.66 vs control: 9.33 ± 4.70).The antibodies elicited bound predominantly to the periacrosomal region of the sperm head and significantly decreased sperm-zona pellucida binding competence *in vitro* (acrolein: 15.48 ± 0.96% vs control: 100 ± 5.59%, $P < 0.0001$). This reduction in fertility was associated with the immunological targeting of several sperm proteins implicated in the mediation of successful conception, including heat shock protein 60, arylsulfatase A, izumo sperm-egg fusion 1 and protein disulfide isomerase A6. Additional studies demonstrated that the enhanced antigenicity of these proteins was highly associated with their selective adduction following direct exposure acrolein *in vitro*. Taken together, these data suggest that acrolein adduction may sufficiently enhance the immunogenicity of the sperm proteins needed to achieve the goal of immunocontraception in mice.

Summary Sentence

Acrolein covalently modifies proteins essential for sperm-egg interaction, which contributes to reduced zona pellucida binding competence following immunization.

Key words: Acrolein, anti-sperm antibodies, electrophilic aldehydes, immunocontraception, infertility, mouse, spermatozoa, vaccine

Introduction

Immunocontraceptives offer a humane alternative to population control methods based on euthanasia, in the context of both domestic and pest animal species [1]. Implementing a method of fertility control that requires only a single administration and persists for the lifetime of the animal is highly desirable [2, 3]. However, the development of a long-acting immunocontraceptives using native proteins has proved a challenging task, largely because of the inability to raise adequate immunity against self antigens, particularly in the male [4]. One strategy that may be used to overcome this deficiency is to induce anti-sperm antibody formation with proteins that have been chemically modified in such a way as to significantly enhance their immunogenicity.

Reactive oxygen species (ROS) play an integral role in both the promotion and suppression of sperm function and are a by-product of normal metabolism, generated largely as a consequence of electron leakage from the sperm mitochondria [5]. However, a delicate balance exists between normal levels of ROS in the cell and the limited scope and restricted localization of antioxidant defenses. Once the latter is overwhelmed a self-perpetuating cascade of ROS generation and lipid peroxidation ensues resulting in the downstream production of a suite of reactive electrophilic aldehydes, including acrolein, 4-hydroxynonenal (4HNE) and malondialdehyde [6]. Spermatozoa are highly susceptible to peroxidative damage due to the prevalence of polyunsaturated fatty acids within the plasma membrane and a lack of cytoplasm that houses antioxidative defense machinery normally found in other cells [7-13]. Following exposure to oxidative stress, sperm commonly

experience perturbation of detoxification mechanisms [13-16] and the formation of covalent adducts on DNA and proteins, culminating in a rapid loss of cell integrity and function [16-20].

ROS has previously been implicated in the accelerated onset of disease pathogenesis [21-25]. Specifically, lipid peroxidation-derived aldehydes are critical in the etiology of autoimmune disease [26]. Indeed, previous studies have demonstrated that aldehyde adduct formation causes vulnerable proteins to undergo conformational modification and become highly immunogenic [21-23, 25, 26]. As the protein is no longer recognized as “self”, a break in immune tolerance occurs and autoimmunity ensues [27].

Electrophilic aldehydes reported to form covalent adducts with proteins during oxidative stress cascades and trigger a state of autoimmunity include acrolein and 4HNE [28, 29]. Given the susceptibility of spermatozoa to oxidative stress and lipid peroxidation, the possibility that aldehyde adduction of sperm proteins essential for fertility might immediately compromise the functionality of these molecules [13, 30] and secondarily induce a state of autoimmunity capable of prolonged eliciting a state of infertility, is a highly novel concept worthy of investigation. Furthermore, if this pathway for inducing a state of immunity against sperm antigens is confirmed there would be clear implications for both the diagnosis of immunological infertility and for the development of an immunocontraceptive vaccine. In humans, heat shock protein A2 (HSPA2), a molecular chaperone necessary for zona pellucida binding competence, is highly vulnerable to adduction following exogenous exposure to 4HNE [30]. Similarly, in stallion spermatozoa, heat shock protein 90 alpha (cytosolic) member A1 (HSP90AA1) and arylsulfatase A (ARSA) are vulnerable to adduction by acrolein and 4HNE, respectively [16]. In mouse spermatozoa, however, a study investigating the impact of exogenous aldehydes, and the use of aldehyde adducted sperm proteins as the basis for a fertility vaccine,

has not been conducted. These considerations provided us with the impetus to explore the consequences of immunizing mice with aldehyde adducted sperm proteins, and thereby provide proof-of-concept data in the mouse that would have implications for fertility regulation in a range of domestic and pest animal species.

Materials and methods

Induction of active immunity

This study was designed to determine whether immunizing mice with sperm proteins covalently modified by electrophilic aldehydes, acrolein and 4HNE, could elicit a contraceptive effect, as measured *in vivo* via a fertility trial and *in vitro* by assessing zona pellucida binding competence. In the first instance, a pilot study was conducted in a small cohort of animals, where both females and males were immunized. For this study eight week old inbred Swiss male and female mice were immunized with 25 µl (400 µg/ml) non-adducted, acrolein-adducted or 4HNE-adducted sperm lysate or 25 µl purified water emulsified in an equal volume of Alhydrogel adjuvant 2% (InvivoGen, San Diego, CA, USA; Figure 1). Booster injections were delivered three weeks after the initial injection. Fertility trials commenced 1 week following the booster delivery by pairing each female with a male within the same treatment group for 5 days. Vaginal plugs were monitored to confirm mating. Female weights were monitored and mice were euthanised 15 days after pairing with the male. Bloods were collected via heart puncture and held at room temperature (RT) for 30 min. Blood was centrifuged at 3000 × *g* for 15 min and the serum was collected and stored at -80°C until use. The uterus was subsequently examined to determine the number of fetuses and implantation scars (Figure 1).

This preliminary trial demonstrated that a reduction in fertility was only observed in the acrolein-adducted immunization group. As a result of this finding we focused

on this particular aldehyde for subsequent immunization trials. In these follow up studies cauda epididymal spermatozoa from immunized mice were subjected to motility analysis via computer-assisted sperm analysis (CASA) as well as zona pellucida binding competence. The presence of anti-sperm antibodies bound to the surface of cauda epididymal spermatozoa or sections of testicular tissue were assessed via immunocyto- and immunohistochemistry, respectively. For the latter, testes were collected, weighed, and stored either at -80°Cfor protein analysis or in Bouin's fixative overnight at 4°C, washed with 70% ethanol and processed for histological analysis. Bloods were collected via heart puncture and serum isolated.

Antibodies

Rabbit polyclonal anti-ACR was obtained from Novus Biologicals (NB200-556; Littleton, CO, USA). Rabbit polyclonal anti-4HNE was obtained from Jomar Diagnostics (HNE11-S; Adelaide, SA, Australia). Rabbit polyclonal anti-HSP60 (sc13966) and goat polyclonal anti-IZUMO1 (sc79543) was obtained from Santa Cruz (Scoresby, VIC, Australia). Rabbit polyclonal anti-PDIA6 (HPA034653) and anti-ARSA (HPA005554) antibodies were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Animal ethics

All experimental procedures were carried out with the approval of the University of Newcastle's Animal Care and Ethics Committee (ACEC; approval number A-2014-405). This research was based on inbred Swiss mice obtained from a breeding colony held at the institute's Central Animal House and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime (16L:8D) at 21–22°C and supplied with food and water *ad libitum*. Animals were euthanized via CO_2 inhalation.

Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). A modified Biggers, Whitten, and Whittingham (BWW) medium [31] containing 95 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 275 µM sodium pyruvate, 3.7 µl/ml 60% sodium lactate syrup, 50 U/ml penicillin, 50 µg/ml streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.1% (w/v) polyvinyl alcohol, with an osmolarity between 290 and 310 mOsm/kg and a pH of approximately 7.4, was utilized throughout this study.

Preparation of spermatozoa for immunization

After euthanasia, the epididymides were dissected out of eight week old inbred Swiss male mice, separating the cauda from fat and overlying connective tissue. Caudal spermatozoa were collected by making tubule incisions and applying gentle pressure to release spermatozoa into BWW medium. Sperm concentration was determined using a NucleoCounter SP-100 (ChemoMetec, Denmark) and diluted to a final concentration of 10×10^6 spermatozoa/ml in BWW. Spermatozoa were left untreated (non-adducted) or treated with 50 µM acrolein or 4HNE at 37°C. Cells were washed in BWW (100 µl aliquots, 3 min at 500 × *g*) and subject to a mild CHAPS (10 mM; BioScientific, Kirrawee, NSW, Australia) extraction containing protease inhibitors (Roche Diagnostics, North Ryde, NSW, Australia) at 4°C for 1 h on constant rotation to remove proteins from the whole spermatozoa. The solution was then centrifuged at 14 000 × *g* for 15 min at 4°C. Protein lysates were quantified using a DC protein assay kit (Bio-Rad Laboratories, Gladesville, NSW, Australia) on a SPECTROstar nano (BMG labtech, Germany) and stored at -20°C until use.

Gel electrophoresis and immunoblotting

Extracted sperm lysates were denatured with sodium dodecyl sulfate (SDS) loading buffer (0.2% SDS, 50% 0.375 M Tris, 10% sucrose, 4% b-mercaptoethanol, and 0.001% bromophenol blue) for 5 min at 100°C. Samples were cooled, loaded onto a 4-20% Mini-PROTEAN TGX precast gel (456-1096; Bio-Rad Laboratories) and separated by electrophoresis at 150 volts for 1 h. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Parramatta, NSW, Australia) using standard Western blot transfer techniques [32]. Nitrocellulose membranes were blocked in 5% skim milk in Tris (0.02 M) and NaCl (0.15 M) TBS containing 0.1% Tween (TBST) at RT for 1 h prior to incubation with vehicle control, acrolein or 4HNE serum from immunized mice or anti -acrolein or -4HNE (1:500) in 1% skim milk in TBST. Immunoblots remained in serum solution overnight at 4°C and were washed three times in TBST before 1 h incubation in goat anti -mouse (1:5000) or –rabbit (1:1000), or mouse anti-goat (1:1000) horseradish peroxidase-conjugated immunoglobulin G secondary antibody in 1% skim milk in TBST. Nitrocellulose membranes were developed using an enhanced chemiluminescence kit (GE Healthcare) as per the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed as described previously [33]. Briefly, slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving slides for 9 min in sodium citrate (10 mM, pH 6). Slides were cooled to RT before being blocked in 3% BSA-TBS (10 mM Tris, pH 7.5, 100 mM NaCl) for 1 h. Sections were incubated with 1:100 Alexa Fluor 594 goat anti-mouse IgG in 1% BSA-TBS for 1 h. Slides were washed three times in TBST for 5 min and then counterstained with 4',6-diamidino-2-phenylindole

(DAPI) for 1 min, rinsed in TBS and mounted in antifade reagent Mowiol (13% Mowiol 4-88, 33% glycerol, 66 mM Tris (pH 8.5), 2.5% 1,4 diazobcyclo-[2.2.2]octane). Images were captured on an Axio Imager A1 fluorescence microscope (Zeiss, North Ryde, NSW, Australia) using cellSens Standard imaging software (Olympus, Notting Hill, VIC, Australia).

Motility analysis

Sperm motility was objectively determined using CASA (IVOS, Hamilton Thorne, Danvers, MA, USA) employing the following settings: negative-phase-contrast optics, recording rate 60 frames/s, minimum contrast 50, minimum cell size four pixels. Cells exhibiting a VAP of ≥ 10 μ/s were considered progressive. A minimum of 200 spermatozoa in five fields were assessed using 80 μm MicroCell slides (Microm, Thame, UK) and a stage temperature of 37°C.

Immunocytochemistry

To assess the presence of antibodies on spermatozoa from immunized mice, live cells were incubated in a 1:100 dilution of goat-anti-mouse Alexa fluor 488 secondary antibody in the dark for 30 min at 37°C. Spermatozoa were washed three times in BWW and mounted onto glass slides. To assess the labeling of proteins of interest, spermatozoa were centrifuged at 500 × *g* for 3 min, washed three times in BWW and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min at 4°C. Cells were then washed three times in 0.1 M glycine in PBS, and permeabilized for 5 min at 4°C in solution consisting of 3.5 mM sodium citrate and 0.1% Triton X-100 in PBS. Cells were then centrifuged at 500 × *g* for 3 min and washed three times in PBS. Spermatozoa were blocked in 3% bovine serum albumin (BSA)/10% goat serum in PBS for 1 h at RT, and primary antibodies applied 1:50 in 1% BSA in PBS overnight at RT. Cells were washed three times in

PBS and centrifuged at 500 × *g* for 3 min and secondary antibodies (anti-mouse, anti-rabbit, anti-goat; 1:200) applied in 1% BSA in PBS for 1 h at RT. Finally, cells were centrifuged at 500 × *g* for 3 min and washed three times in PBS. Cells were counterstained with DAPI, washed and mounted. For all immunocytochemical analyses, images were captured on an Axio Imager A1 fluorescence microscope, and secondary antibody only controls were utilized to monitor non-specific binding.

Zona pellucida binding assay

Oocytes were collected from six week old inbred Swiss mice as described previously [34]. Briefly, mice were superovulated by administering 10 IU pregnant mare gonadotrophin (PMSG) and 10 IU human chorionic gonadotrophin (hCG) 48 h later, both via intraperitoneal injection. Approximately 15 h after hCG administration, the mice were euthanized and the oocyte cumulus complexes recovered and placed in 0.1% hyaluronidase for 15 min at 37°C. Oocytes were separated from adherent cumulus cells by gentle pipetting and washed three times in BWW. Oocytes were stored at 4°C in a high salt storage medium consisting of 1.5 mM $MgCl_2$, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% polyvinyl alcohol until used.

Salt stored oocytes were washed three times in PBS and eight oocytes per treatment were placed in droplets of BWW under water-saturated mineral oil at 37°C in an atmosphere of 5% CO_2 : 95% air and allowed to equilibrate for 30 min. The zona pellucida binding assay was performed similarly to that described by Bromfield, et al. [30]. Spermatozoa from immunized mice were capacitated for 45 min in BWW media supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic adenosine monophosphate. Cells were incubated at 37°C under an atmosphere of 5% CO_2 : 95% air. Non-capacitated cells were incubated for the same period of time in BWW prepared without $NaHCO_3$. Spermatozoa were diluted to a concentration of 1×10^6 cells/ml and 20 µl of sperm suspension was added to

each droplet of oocytes. Gametes were co-incubated under the same conditions for a further 30 min. Oocytes were then washed by serial aspiration through droplets of BWW to remove any unbound sperm, and mounted on glass slides under coverslips suspended at each corner by 80% paraffin wax and 20% vaseline gel. The number of motile sperm bound to each zona pellucida was assessed using phase contrast microscopy.

Mass spectrometry

Unique protein bands were excised and identified using a one-dimensional liquid chromatography electrospray ionization mass spectrometry/mass spectrometry (1D LC ESI MS/MS) interface at the Australian Proteome Analysis Facility (APAF). Membranes were incubated in 0.5% (w/w) polyvinylpyrrolidine in 100 mM acetic acid for 30 min at 37°C. Membrane washed six times with 500 µl water at 37°C. Proteins were digested with 250 ng trypsin in 100 mM ammonium bicarbonate overnight. Sample (10 µl) was injected onto a peptide trap (Halo C18, 150 µm x 5 cm) for pre-concentration and desalted with 0.1% formic acid, 2% ACN at 4 µl/min for 10 min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O:CH_3CN (98:2; + 0.1% formic acid) to H_2O:CH_3CN (2:98; + 0.1% formic acid) with constant flow (600 nl/min) over an 80 min period. The LC eluent was subject to positive ion nanoflow electrospray MS analysis in an information dependant acquisition mode (IDA). In the IDA mode a TOFMS survey scan was acquired (m/z 350-1500, 0.25 sec), with twenty largest multiply charged ions (counts >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 milli-seconds (m/z 100-1500) with rolling collision energy. The raw data files (.wiff) were converted to mascot generic files (.mgf) using AB SCIEX CommandDriver software. They were submitted to Mascot (Matrix Science,

UK) and searched against *Mus musculus* in the SwissProt database. The search was performed with a false-discovery rate of <1% with score cut-off of 47 applied. Deamidated (N), deamidated (Q) and oxidation (M) were set as variable modifications.

Immunoprecipitation

Cell lysis of acrolein-adducted sperm proteins was performed on 100 × 10^6 cells at 4°C for 2 h in IP lysis buffer consisting of 10 mM CHAPS, 10 mM HEPES, 137 mM NaCl and 10% glycerol with a protease inhibitor (Roche), as described previously [16]. Cells were centrifuged at 14 000 × *g* at 4°C for 20 min, and added to 50 µl aliquots of washed protein G Dynabeads to preclear at 4°C for 1 h under constant rotation. Meanwhile, 10 µg of target antibody was added to protein G Dynabeads and incubated at 4°C for 2 h under constant rotation before undergoing cross-linking using 5 mM bis(sulfosuccinimidyl)suberate in 20 mM HEPES. The antibody-bound protein G Dynabeads were then resuspended in the total volume of precleared lysate, and co-incubated overnight at 4°C under constant rotation. Following this, antibody-antigen-bound beads were washed three times in PBS before being resuspended in SDS loading buffer and incubated at 100°C for 5 min to elute proteins. The eluted protein was loaded onto a 4–20% gel (Bio-Rad Laboratories) along with antibody only, bead only and precleared controls.

Statistical analyses

All experiments were replicated at least three times on independent samples. Datasets were checked for normality using the Shapiro-Wilk. Data appeared normal and were consequently analyzed by ANOVA using JMP11 program (SAS Institute, Raleigh, NC, USA) with Dunnett's test. Differences with a P-value < 0.05 were considered significant.

Results

Fertility is reduced in mice following immunization with acrolein-adducted sperm proteins

To assess whether the generation of anti-sperm antibodies against aldehyde modified sperm lysates was sufficient to elicit changes to fertility, both female and male mice were immunized and reproductive outcomes assessed. A modest reduction in fertility, as assessed by counting the number of fetuses in the uterus, was apparent in females immunized with a cocktail of proteins extracted from acrolein treated spermatozoa following mating with immunized males (Figure 2A). In two of the three females immunized with acrolein-adducted sperm proteins, no conceptions occurred; however, one female failed to mount an immune response sufficient to impede fertility, as visualized via immunoblotting (Figure 2B). The antibody profile of an acrolein-adducted sperm protein immunized mouse that fell pregnant shows a very weak antibody response, while the two mice that did not fall pregnant had significant antibodies generated against a 60 kDa band and a series of high molecular weight sperm proteins (130 – 200 kDa). In contrast to the acrolein-adducted immunization group, there was no decrease in the number of fetuses from mice immunized with 4HNE-adducted sperm proteins.

Glucose-6-phosphate isomerase, arylsulfatase A, izumo sperm-egg fusion 1 and protein disulfide isomerases are targets for covalent modification by acrolein in mouse spermatozoa in vitro

Electrophilic aldehyde treated sperm lysates were subjected to immunoblotting with the corresponding anti-aldehyde antibody to visualize the molecular weights of adducted proteins (Figure 3). Acrolein adduction was predominantly associated with a protein band of 60 kDa, whereas 4HNE adduction occurred on a protein band of

38 kDa. Additional aldehyde adducted bands, albeit of lower staining intensity, were consistently detected across the range of 35 kDa to 200 kDa. The predominant bands were excised and identified using LC–MS/MS. The 60 kDa band taken from the acrolein immunoblot revealed a shortlist of proteins necessary for sperm-egg binding and fusion, including glucose-6-phosphate isomerase (G6PI), arylsulfatase A (ARSA), izumo sperm-egg fusion 1 (IZUMO1), and protein disulfide isomerases (PDIA3, PDIA1, PDIA6; Table 2). Comparatively, the 38 kDa 4HNE band did not contain any proteins which were relevant to fertility (Supplementary table 1), potentially explaining why there was no contraceptive effect when mice were immunized with 4HNE-adducted sperm proteins. Consequently, immunizations with acrolein-adducted sperm proteins became the focus of our study.

Antibodies generated in response to active immunization with acrolein-adducted sperm proteins bind to the male germ line and impact sperm function

Although testis sections from mice immunized with acrolein-adducted sperm proteins did not reveal any major changes to testicular morphology (Figure 4), immunocytochemistry revealed the presence of antibodies bound to elongating spermatids and spermatozoa. The fluorescence signal was most pronounced in tissue sections taken from mice immunized with acrolein-adducted sperm proteins, suggesting that the immunization effectively stimulated the production of antibodies which recognized and bound to sperm proteins. Fluorescence was less pronounced in tissue sections obtained from mice immunized with non-adducted proteins and no staining was evident in vehicle control tissues.

Motility analysis of sperm taken from immunized mice revealed no changes between treatment groups (Figure 5A). Immunocytochemical analysis of sperm from mice immunized with acrolein-adducted sperm proteins revealed antibodies generated against proteins along the periacrosomal region of the head (Figure 5B). To determine whether the presence of such antibodies was of functional

significance, analyses of zona pellucida binding competence was performed. The ability of spermatozoa to bind to the zona pellucida of salt stored oocytes was significantly inhibited, when compared to the capacitated control ($P < 0.001$; Figure 5C). Although there was a significant decrease between spermatozoa isolated from the vehicle control and those recovered from mice immunized with non-adducted sperm proteins ($P < 0.01$), this was significantly exacerbated by the adduction of sperm proteins with acrolein prior to immunization.

Antibodies are generated against glucose-6-phosphate isomerase, arylsulfatase A, izumo sperm-egg fusion 1, protein disulfide isomerases and heat shock protein 60 following immunization with acrolein-adducted sperm proteins

Acrolein-adducted sperm lysates were subjected to immunoblotting and probed with either blood serum from vehicle control or acrolein-adducted immunized mice (Figure 6). In each instance a similar protein profile occurred on immunoblots probed with vehicle control serum compared to acrolein-adducted sperm protein immunized mice serum. In vehicle control serum, a band at 60 kDa was either not evident or was lowly expressed. In contrast, a strong band occurred at 60 kDa in each of the acrolein-adducted serum replicates, mirroring the results of the analysis of acrolein-adducted sperm proteins depicted in Figure 3.

To determine the identity of the 60 kDa proteins, the band was excised and subjected to nano-LC tandem mass spectrometry (LC–MS/MS). This approach led to high confidence identification of glucose-6-phosphate isomerase (G6PI), izumo sperm-egg fusion 1 (IZUMO1), heat shock protein 60 (HSP60), arylsulfatase A (ARSA), and protein disulfide isomerases (PDIA3, PDIA1, PDIA6; Table 3), mirroring the cohort of proteins that are preferentially adducted by acrolein *in vitro*.

Immunoprecipitation of acrolein-adducted proteins was performed and the precipitates subsequently probed with antibodies against HSP60, ARSA, IZUMO1

and PDIA6 in order to confirm acrolein adduction of these proteins in treated spermatozoa (Figure 7A). Reciprocal pulldowns were then conducted, pulling down each of the proteins of interest and probing the precipitates for the presence of acrolein (Figure 7B). We next assessed the protein labeling of HSP60, ARSA, IZUMO1 and PDIA6 via immunocytochemistry (Figure 7C). In fixed and permeabilized spermatozoa, each protein had varying levels of labeling across the periacrosomal region of the head. HSP60 also labeled the proximal centriole, ARSA a region of the mid piece, and IZUMO1 was less intensely labeling in other regions of the head, mid piece and principle piece of the tail. Finally, PDIA6 was also labeled along the mid piece. In each instance, protein labeling colocalized to regions in which acrolein preferentially accumulates within the cell. Collectively, these data confirm that HSP60, ARSA, IZUMO1 and PDIA6 are covalently modified by acrolein binding in mouse spermatozoa.

To confirm that the observed antigenic targets were located in the same region of the male tract as the auto-antibodies were localized (Figure 4), antibodies against one of these candidate proteins (PDIA6) were used to stain testicular sections. Anti-PDIA6 generated weak labeling across the entire testicular cross section, similar to findings in the human testis [35], but was predominantly expressed in the heads of elongating spermatids, localizing to the same target sites as the antibodies generated following immunization.

Discussion

Spermatozoa are highly susceptible to lipoperoxidative damage, making them vulnerable to the formation of lipid aldehyde adducts on key proteins necessary for successful fertilization. The aim of this study was to determine the contraceptive potential of mice immunized with sperm protein lysate previously treated with electrophilic aldehydes, acrolein and 4HNE. Our findings indicate that exposure to

acrolein-, but not 4HNE-, adducted proteins induced the formation of antibodies that were capable of reducing the zona pellucida binding competence of spermatozoa *in vitro* via mechanisms that did not influence sperm motility. The induction of active immunity against acrolein-adducted sperm proteins also suppressed fertility *in vivo*, suggesting that this approach holds promise for the development of immunocontraceptive reagents targeting the functionality of the male gamete.

Analysis of the acrolein adduction profile revealed a surprisingly restrictive pattern of adduction with a major band of proteins being targeted that had an aggregate molecular mass of around 60 kDa. Excision of this band, followed by mass spectrometry, revealed a shortlist of proteins modified by acrolein including: (1) glucose-6-phosphate isomerase (G6PI), a key enzyme in the glycolytic pathway of energy metabolism, (2) arylsulfatase A (ARSA), implicated in mediation of sperm-zona pellucida recognition/adhesion, (3) izumo sperm-egg fusion 1 (IZUMO1), a mediator of sperm–oocyte fusion and (4) protein disulfide isomerases (PDIA3, PDIA1, PDIA6), mediators of protein folding that are also associated with sperm-egg fusion. Conversely, following incubation of spermatozoa with 4HNE *in vitro*, aldehyde adducts occurred on a ~38 kDa band revealing proteins with no direct association to sperm-egg interaction (Supplementary table 1). This finding is somewhat unique as the impact of exogenous 4HNE on spermatozoa from other species, such as the human and horse, has been shown to be responsible for modifying sperm-egg interacting proteins, heat shock protein A2 (HSPA2) [30] and ARSA [16], respectively. In a recent study by Bromfield, et al. [36] proteolytic degradation of HSPA2 occurred in round spermatids of mice and a murine spermatogenic cell line following exogenous treatment with 4HNE, at a dose and exposure time consistent with this study. However, the impact of 4HNE on cauda epididymal spermatozoa and subsequent HSPA2 expression was not assessed. This omission leaves open the possibility that 4HNE preferentially targets proteins in

precursor germ cells in the mouse; by the time spermiogenesis and epididymal maturation have occurred, the sites available for adduction by 4HNE no longer influence sperm function.

The impact of 4HNE on spermatozoa has been studied extensively in a variety of species [16, 17, 30, 37] since it is the most abundant aldehyde produced following lipoperoxidative damage [37-39]. In comparing the identity of proteins adducted by lipid aldehydes in different species, it should also be noted that the second order rate constants describing the reactivity of lipid aldehydes with a model thiol (glutathione) demonstrate that acrolein is a significantly more reactive aldehyde than 4HNE, reflecting its greater bioactivity [37]. Such differences in reactivity may go some way towards explaining the different spectrum of proteins targeted by 4HNE and acrolein in the mouse and could explain the former's inability to form adducts capable of eliciting the formation of antibodies that suppress fertility.

The consistent manner in which ARSA is targeted for electrophilic aldehyde adduction in different species is worthy of comment. In this study, ARSA was adducted by acrolein, while in equine spermatozoa 4HNE was capable of ARSA adduction. In human spermatozoa, ARSA plays a quintessential role in the HSPA2-ARSA-SPAM1 molecular chaperone complex responsible for sperm–egg recognition. This complex is disrupted in human spermatozoa, and sperm-zona pellucida binding is suppressed because of the ability of 4HNE to form a stable adduct not with ARSA but with the molecular chaperone HSPA2 [30]. Whilst direct (horse; [16]) or indirect (human; [30]) disruption of ARSA function suppresses sperm-zona pellucida binding, the same cannot be said of mouse spermatozoa. While ARSA was clearly targeted by acrolein in the mouse spermatozoa, and fully motile sperm from mice actively immunized against acrolein-adducted proteins lost their capacity to bind to the zona pellucida, the fact remains that the ARSA knockout mouse (*Arsa*$^{-/-}$) is fertile, even though the accumulation of sulfogalactosylglycerolipid

in the spermatozoa of such genetically–modified mice places limits on their reproductive life span [38]. These findings suggest a genuine functional difference between mice and other species in the molecular basis of sperm-egg recognition.

The one aspect of sperm–egg recognition that is established in the mouse is the central involvement of HSP60 [34]. It is conceivable that, as in human spermatozoa, a molecular chaperone complex exists between HSP60 and other sperm-egg interacting proteins and following adduction the function of this particular molecular chaperone complex is compromised. In addition to HSP60, Asquith, et al. [34] also reported ERp99 phosphorylation upon capacitation, which is a known homologue of HSP90 [39] and a target for acrolein adduction in stallion spermatozoa [16]. Excitingly, Pires, et al. [40] have shown that modification of HSP90 through autoimmunization or aldehyde adduction modification alters the normal protein structure which is necessary for successful sperm-egg interactions. Certainly, different proteins appear vulnerable to adduction by electrophilic aldehydes between species; however, consistent between species is that the adduction process appears to be restricted to a subset of sperm proteins largely involved in sperm-egg interaction as well as ATP generation which is necessary for flagellar movement [13, 17]. Both aspects are critical for successful fertilization, which would suggest a conserved process occurs to prevent sperm that potentially bare oxidatively damaged DNA from participating in fertilization, and thus perpetuating such damage to future offspring.

In the zona pellucida binding studies, spermatozoa from mice immunized with non-adducted proteins responded less dramatically than acrolein-adducted; however, a slight but significant decrease was observed. Regardless of this outcome, the fertility trial revealed no major changes to the number of fetuses in pregnant females immunized with non-adducted sperm proteins, while the induction of immunity with acrolein-adducted proteins generated a clear contraceptive effect.

This suggests that despite the fact that a reduced number of spermatozoa were able to bind to the zona pellucida *in vitro*, there were still sufficient numbers of functional sperm to fertilize each oocyte *in vivo* when mice were immunized with non-adducted sperm proteins. This observation supports the hypothesis that modified proteins would elicit a larger immune response than non-modified proteins. Indeed, the serum from females immunized with acrolein-adducted sperm proteins that did not fall pregnant showed a group of high molecular weight bands (visualized via immunoblotting) that were not present in a replicate that failed to establish an immune response, nor a contraceptive response (Figure 2). This further suggests the presence of antibodies against sperm proteins in the female may have played an equal role to the male in the preventing pregnancy in this study.

The binding of acrolein to IZUMO and the subsequent generation of antibodies against this protein is of interest because a significant percentage of infertile women have been shown to possess antibodies against the IZUMO protein [41]. Anti-IZUMO antibodies could not however, explain the lack of sperm-zona pellucida binding observed as a consequence of the induction of immunity against the acrolein-adducted sperm proteins because this protein (like the PDIs also identified in this study) is primarily involved in sperm-oocyte fusion rather than sperm-egg recognition. Nevertheless, since functional deletion of IZUMO1 in knockout mice does lead to infertility [42], it is possible that this sperm protein is involved in the infertility observed following the induction of active immunity with against sperm proteins complexed with acrolein.

The molecular basis for the loss of sperm-zona pellucida binding activity is less clear. As indicated above, ARSA cannot be indispensable for this process to occur because ARSA knockout mice are fertile [38]. By analogy with the sperm-zona pellucida recognition in human spermatozoa it is possible that the adduction of a molecular chaperone is critically involved in the disruption of sperm-zona pellucida

binding. In human spermatozoa, this process is mediated by a molecular complex involving HSPA2, SPAM1 and ARSA [43]. In the mouse, sperm-zona pellucida recognition may be mediated by a complex involving HSP60 [34] and other, as yet uncharacterized molecules [44]. Identification of these proteins that associate with HSP60 will be a high priority task for the future, as HSP60 itself is too widely distributed to constitute a reasonable target for immunocontraception. However, the proteins that associate with HSP60 to create a functional sperm-zona pellucida recognition complex may make ideal candidates for a fertility regulating vaccine targeting the process of conception.

The findings presented in this study demonstrate that immunization with acrolein-adducted sperm proteins generates antibodies against a series of proteins which are essential for successful conception, providing a proof-of-concept for future attempts at fertility control. Certainly in other species such as the horse in which similar proteins have been targeted following exogenous aldehyde exposure [16], this approach may be readily transferred for the development of a novel immunocontraceptive to manage feral horses. Additionally, despite that during this study there was little effect of immunizations with 4HNE-adducted sperm proteins in the mouse, it is still worthwhile pursuing 4HNE-adducted immunizations for species whose spermatozoa have previously been shown to have proteins which are susceptible to 4HNE adduction.

Acknowledgment

Mass spectrometry work was conducted at APAF. The authors thank Nathan D. Smith for technical assistance in mass spectrometry sample preparation and analysis.

REFERENCES

1. Hardy CM, Braid AL. Vaccines for immunological control of fertility in animals. Rev Sci Tech 2007; 26:461-470.
2. Levy JK. Contraceptive vaccines for the humane control of community cat populations. Am J Reprod Immunol 2011; 66:63-70.
3. Hall SE, Nixon B, Aitken RJ. Non-surgical sterilisation methods may offer a sustainable solution to feral horse (Equus caballus) overpopulation. Reprod Fertil Dev 2016:10.1071/RD16200.
4. Kutzler M, Wood A. Non-surgical methods of contraception and sterilization. Theriogenology 2006; 66:514-525.
5. Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal 2011; 14:367-381.
6. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 1998; 111 (Pt 5):645-656.
7. Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med 2010; 48:112-119.
8. Aitken RJ, Baker MA. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. Int J Dev Biol 2013; 57:265-272.
9. Aitken RJ, Wingate JK, De Iuliis GN, Koppers AJ, McLaughlin EA. Cis-unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. J Clin Endocrinol Meta 2006; 91:4154-4163.

10. De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. Biol Reprod 2009; 81:517-524.
11. Aitken R, De Iuliis G. On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod 2010; 16:3-13.
12. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 1979; 31:531-537.
13. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. J Biol Chem 2012; 287:33048-33060.
14. Gibb Z, Lambourne SR, Curry BJ, Hall SE, Aitken RJ. Aldehyde Dehydrogenase Plays a Pivotal Role in the Maintenance of Stallion Sperm Motility. Biol Reprod 2016; 94:133.
15. Carbone DL, Doorn JA, Petersen DR. 4-Hydroxynonenal regulates 26S proteasomal degradation of alcohol dehydrogenase. Free radical biology & medicine 2004; 37:1430-1439.
16. Hall SE, Aitken RJ, Nixon B, Smith ND, Gibb Z. Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa. Biol Reprod 2017:bio145292.
17. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be

significantly rescued by the presence of nucleophilic thiols. Biol Reprod 2012; 87:110.

18. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev 2015; 28:1-10.

19. Hughes LM, Griffith R, Carey A, Butler T, Donne SW, Beagley KW, Aitken RJ. The spermostatic and microbicidal actions of quinones and maleimides: toward a dual-purpose contraceptive agent. Mol Pharmacol 2009; 76:113-124.

20. Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 1987; 81:459-469.

21. Kurien BT, Scofield RH. Autoimmunity and oxidatively modified autoantigens. Autoimmun Rev 2008; 7:567-573.

22. Wang G, Pierangeli SS, Papalardo E, Ansari GAS, Khan MF. Markers of oxidative and nitrosative stress in systemic lupus erythematosus: Correlation with disease activity. Arthritis Rheum 2010; 62:2064-2072.

23. Khan MF, Wu X, Ansari GA. Anti-malondialdehyde antibodies in MRL+/+ mice treated with trichloroethene and dichloroacetyl chloride: possible role of lipid peroxidation in autoimmunity. Toxicol Appl Pharmacol 2001; 170:88-92.

24. Hadjigogos K. The role of free radicals in the pathogenesis of rheumatoid arthritis. Panminerva Med 2003; 45:7-13.

25. Kurien BT, Scofield RH. Free radical mediated peroxidative damage in systemic lupus erythematosus. Life Sci 2003; 73:1655-1666.

26. Wang G, Li H, Firoze Khan M. Differential oxidative modification of proteins in MRL+/+ and MRL/lpr mice: increased formation of lipid peroxidation-

derived aldehyde-protein adducts may contribute to accelerated onset of autoimmune response. Free Radic Res 2012; 46:1472-1481.

27. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem 2008; 283:21837-21841.

28. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991; 11:81-128.

29. Uchida K, Stadtman E. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. Proceedings of the National Academy of Sciences 1992; 89:4544-4548.

30. Bromfield EG, Aitken RJ, Anderson AL, McLaughlin EA, Nixon B. The impact of oxidative stress on chaperone-mediated human sperm–egg interaction. Hum Reprod 2015:dev214.

31. Biggers J, Whitten W, Whittingham D. The culture of mouse embryos in vitro. In: Daniels J (ed.) Methods in Mammalian Embryology. San Fransisco: Freeman; 1971: 86-116.

32. Koppers A, Mitchell L, Wang P, Lin M. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. Biochem J 2011; 436:687-698.

33. Sutherland J, Keightley R, Nixon B, Roman SD, Robker RL, Russell DL, McLaughlin E. Suppressor of cytokine signaling 4 (SOCS4): Moderator of ovarian primordial follicle activation. J Cell Physiol 2012; 227:1188-1198.

34. Asquith KL, Baleato RM, McLaughlin EA, Nixon B, Aitken RJ. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. J Cell Sci 2004; 117:3645-3657.

35. Bromfield EG, McLaughlin EA, Aitken RJ, Nixon B. Heat Shock Protein member A2 forms a stable complex with angiotensin converting enzyme and protein disulfide isomerase A6 in human spermatozoa. Mol Hum Reprod 2015:gav073.

36. Bromfield EG, Aitken RJ, McLaughlin EA, Nixon B. Proteolytic degradation of heat shock protein A2 occurs in response to oxidative stress in male germ cells of the mouse. Mol Hum Reprod 2016.

37. Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, Aitken RJ. Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. Mol Hum Reprod 2015; 21:502-515.

38. Xu H, Kongmanas K, Kadunganattil S, Smith CE, Rupar T, Goto-Inoue N, Hermo L, Faull KF, Tanphaichitr N. Arylsulfatase A deficiency causes seminolipid accumulation and a lysosomal storage disorder in Sertoli cells. J Lipid Res 2011; 52:2187-2197.

39. Mazzarella RA, Green M. ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). J Biol Chem 1987; 262:8875-8883.

40. Pires ES, Choudhury AK, Idicula-Thomas S, Khole VV. Anti-HSP90 autoantibodies in sera of infertile women identify a dominant, conserved epitope EP6 (380-389) of HSP90 beta protein. Reprod Biol Endocrinol 2011; 9:16.

41. Naz RK. Vaccine for human contraception targeting sperm Izumo protein and YLP12 dodecamer peptide. Protein Sci 2014; 23:857-868.

42. Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 2005; 434:234-238.
43. Redgrove KA, Anderson AL, McLaughlin EA, O'Bryan MK, Aitken RJ, Nixon B. Investigation of the mechanisms by which the molecular chaperone HSPA2 regulates the expression of sperm surface receptors involved in human sperm–oocyte recognition. Mol Hum Reprod 2013; 19:120-135.
44. Avella MA, Xiong B, Dean J. The molecular basis of gamete recognition in mice and humans. Mol Hum Reprod 2013; 5:279-289.

FIGURE LEGENDS

Figure 1. *In vivo* experimental design. Mouse spermatozoa were left untreated, or treated with 50 µM acrolein or 50 µM 4HNE for 3 h at 37°C before undergoing a CHAPS extraction to isolate surface proteins. Protein lysate was then delivered to mice with an equal volume of adjuvant via a subcutaneous immunization (treatment groups: vehicle control, non-adducted, acrolein-adducted and 4HNE-adducted). Booster immunizations were delivered 3 weeks later. One cohort of female mice (n = 9) and three cohorts of male mice (n = 36) were immunized with n = 3 in each treatment group. Fertility trials commenced 1 week following the booster delivery by pairing each female with a male within the same treatment group for 5 days. The uterus from female mice was examined to determine the number of fetuses and implantation scars. The testes from immunized males were fixed and sectioned for histological analysis and immunohistochemistry. Sperm was extracted from the cauda epididymis and assessed for motility parameters via CASA,

immunocytochemistry and zona pellucida binding competence. Blood serum from both males and females was collected for use as a probe in immunoblotting.

Figure 2. A) Number of fetuses in a fertility trial after mating male and female mice immunized with aldehyde-adducted sperm proteins. **B**) Western blot analyses of sperm protein lysates probed with serum from each of the acrolein female mice which were pregnant with 14, 0 and 0 fetuses, respectively. Loading control tubulin.

Figure 3. Western blot analyses of sperm protein lysates following exposure to 50 μM acrolein or 50 μM 4HNE at 37°C for 3 h. Each lane represents a different mouse. Loading control tubulin.

Figure 4. Top panel reveals histological testis sections from immunized mice, while the bottom two panels show fluorescent antibody labeling (Alexa Fluor 594; red, DAPI; blue) on elongating spermatids. Images are representative of all replicates (n = 6).

Figure 5. A) Motility of mouse spermatozoa following immunization. Data correspond to mean values ± SEM. **B**) Immunolabeling of live spermatozoa revealed antibody labeling (Alexa Fluor 488; green) on the periacrosomal region of the head in sperm from mice immunized with acrolein-adducted sperm proteins. Images are representative of all replicates (n = 6). **C**) Zona pellucida binding competence following immunization. Non-capacitated cells were used as a negative control. Data correspond to mean values ± SEM as a percentage of the vehicle control; n = 6. **P, < 0.01, ***P, < 0.001 for differences compared with the vehicle control.

Figure 6. Western blot analysis of sperm protein lysates from mice probed with either **A**) serum from a vehicle control male or **B**) serum from a male mouse previously immunized with acrolein-adducted sperm lysate.

Figure 7. Reciprocal pull-downs and IP of **A**) acrolein and **B**) HSP60, ARSA, IZUMO1 and PDIA6. **C**) Fixed and permeabilized spermatozoa labeled with antibodies against proteins of interest (Alexa Fluor 488; green) following 50 μM aldehyde exposure at 37°C for 3 h. Images are representative of all replicates (n = 3).

Figure 8. Anti-PDIA6 labeling (Alexa Fluor 488; green) on testicular sections colocalized with DAPI nuclear stain. Images are representative of all replicates (n = 3).

TABLE 1. Antibodies used throughout the duration of the study

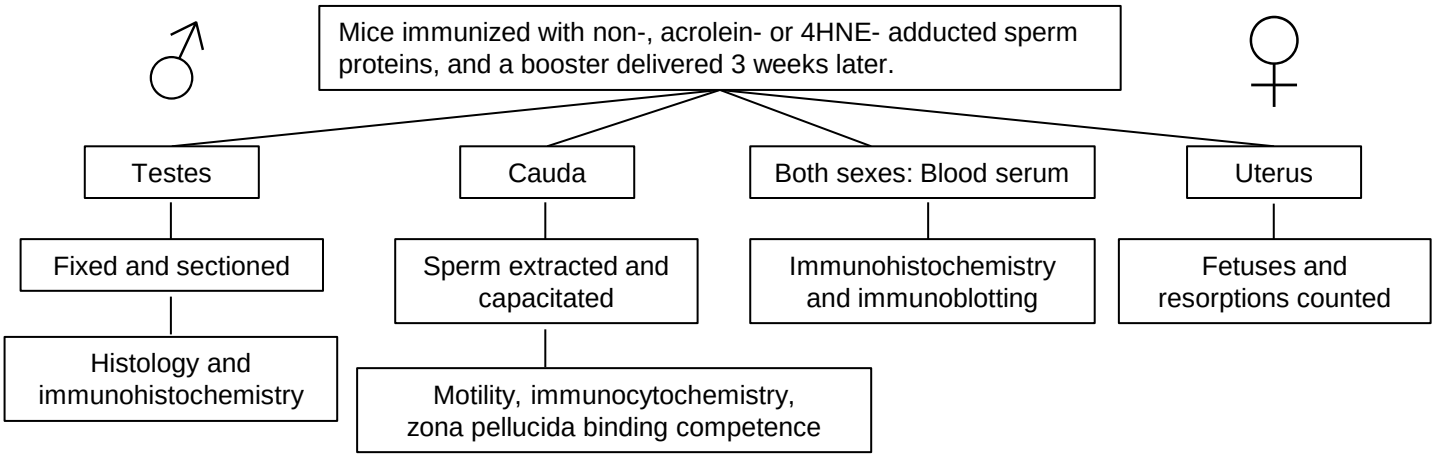
Antibody	Raised	Catalogue	Distributor
Acrolein	Rabbit	NB200-556	Novus Biologicals
4-hydroxynonenal	Rabbit	HNE11-S	Jomar Diagnostics
Heat shock protein 60	Rabbit	sc13966	Santa Cruz
Izumo sperm-egg fusion 1	Goat	sc79543	Santa Cruz
Protein disulfide isomerase A6	Rabbit	HPA034653	Sigma-Aldrich
Arylsulfatase A	Rabbit	HPA005554	Sigma-Aldrich

TABLE 2. Identification of potential targets for acrolein adduction *in vitro*				
Protein (symbol)	Mascot score	Peptides matches	Peptide sequences	UniProt accession number
Glucose-6-phosphate isomerase	944	37	11	P06745
Protein disulfide-isomerase A3 (PDIA3)	685	19	12	P27773
Protein disulfide-isomerase A1 (PDIA1)	561	14	9	P09103
Izumo sperm-egg fusion protein 1 (IZUMO1)	121	4	3	Q9D9J7
Arylsulfatase A (ARSA)	75	6	4	P50428
Protein disulfide-isomerase A6 (PDIA6)	61	1	1	Q922R8

TABLE 3. Identification of potential targets for acrolein adduction *in vivo*				
Protein (symbol)	Mascot score	Peptides matches	Peptide sequences	UniProt accession number
Glucose-6-phosphate isomerase (G6PI)	1396	66	19	P06745
Protein disulfide-isomerase A3 (PDIA3)	1113	43	22	P27773
Protein disulfide-isomerase A1 (PDIA1)	601	32	21	P09103
Izumo sperm-egg fusion protein 1 (IZUMO1)	408	23	10	Q9D9J7
60 kDa heat shock protein (HSP60)	396	17	13	P63038
Arylsulfatase A (ARSA)	137	12	7	P50428
Protein disulfide-isomerase A6 (PDIA6)	84	3	2	Q922R8

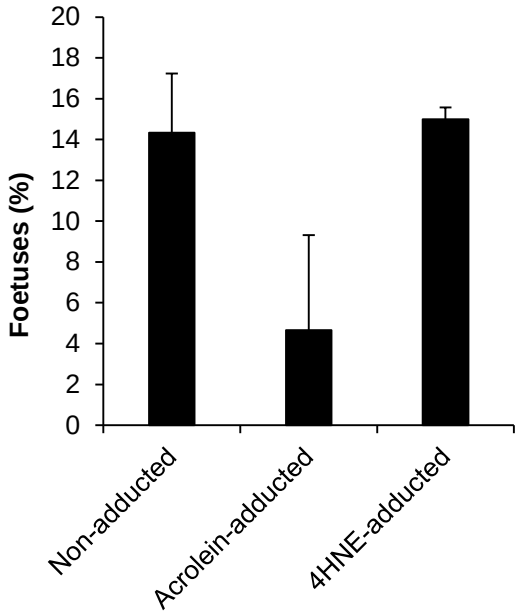
Supplementary table 1. Identification of potential targets for 4HNE adduction *in vitro*				
Protein (symbol)	Mascot score	Peptides matches	Peptide sequences	UniProt accession number
Aspartate aminotransferase (AATM)	528	21	10	P05202
Phosphoglycerate kinase 2 (PGK2)	467	17	9	P09041
Solute carrier family 2, facilitated glucose transporter member 3 (GTR3)	308	10	5	P32037
Actin, cytoplasmic 1 (ACTB)	298	11	6	P60710
Sorbitol dehydrogenase (DHSO)	283	11	5	Q64442
Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial (SUCB1)	69	3	3	Q9Z2I9
L-lactate dehydrogenase A chain (LDHA)	58	1	1	P06151
Solute carrier family 2, facilitated glucose transporter member 5 (GTR5)	56	3	2	Q9WV38

1

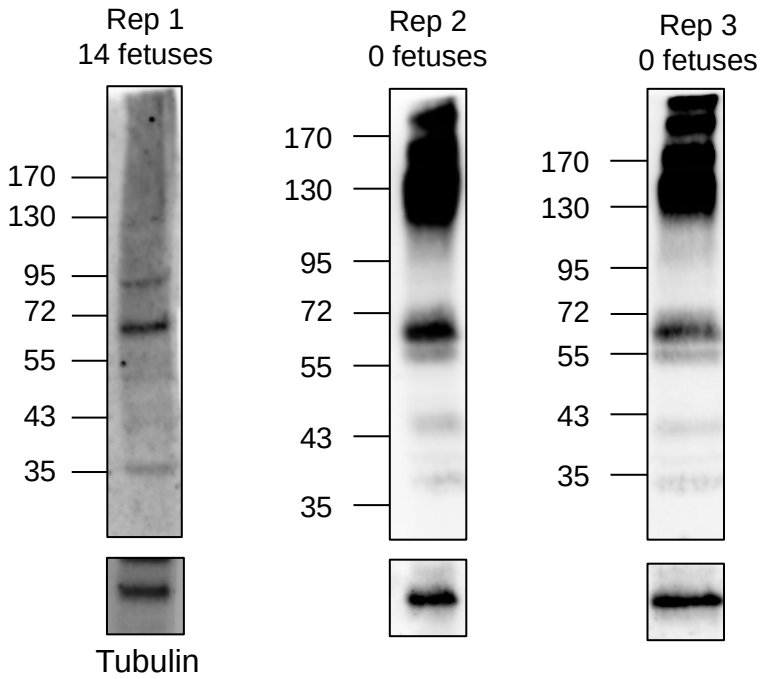

Mice immunized with non-, acrolein- or 4HNE- adducted sperm proteins, and a booster delivered 3 weeks later.
Testes
Cauda
Both sexes: Blood serum
Uterus
Fixed and sectioned
Sperm extracted and capacitated
Immunohistochemistry and immunoblotting
Fetuses and resorptions counted
Histology and immunohistochemistry
Motility, immunocytochemistry, zona pellucida binding competence

2

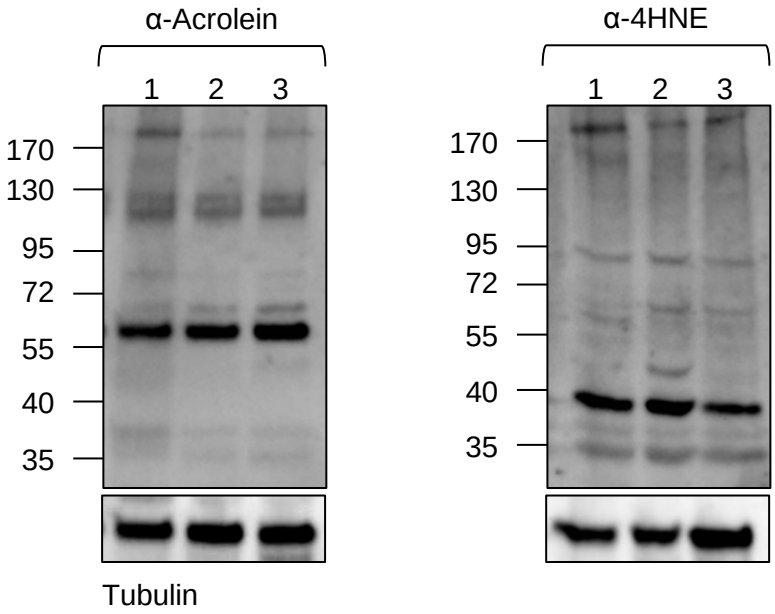
A


Foetuses (%)
20
18
16
14
12
10
8
6
4
2
0
Non-adducted
Acrolein-adducted
4HNE-adducted

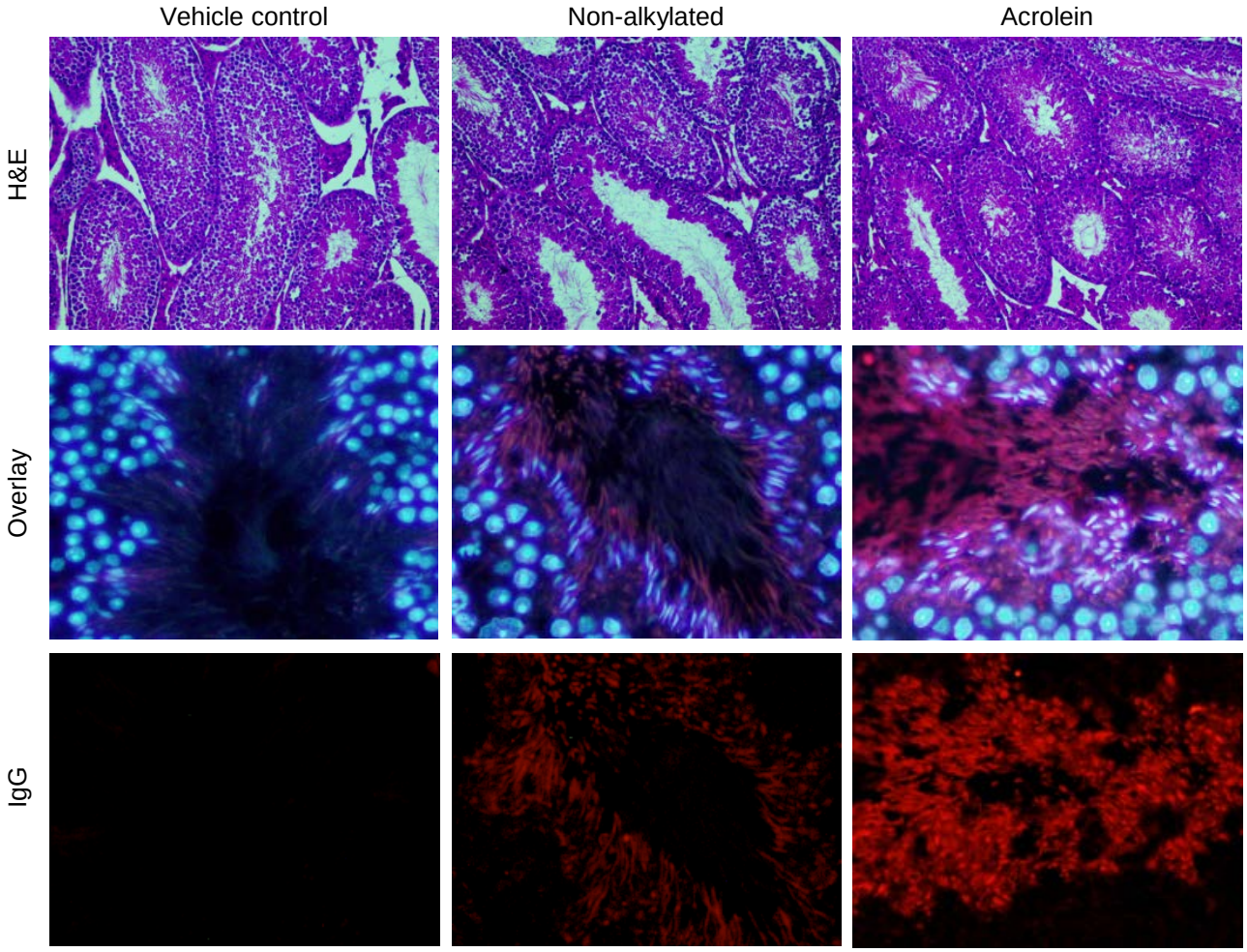
B


Rep 1
14 fetuses
Rep 2
0 fetuses
Rep 3
0 fetuses
170
130
95
72
55
43
35
Tubulin

3

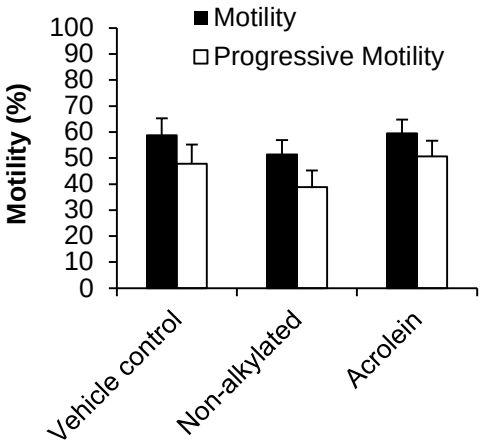

α-Acrolein
1
2
3
170
130
95
72
55
40
35
Tubulin
α-4HNE
1
2
3
170
130
95
72
55
40
35

4

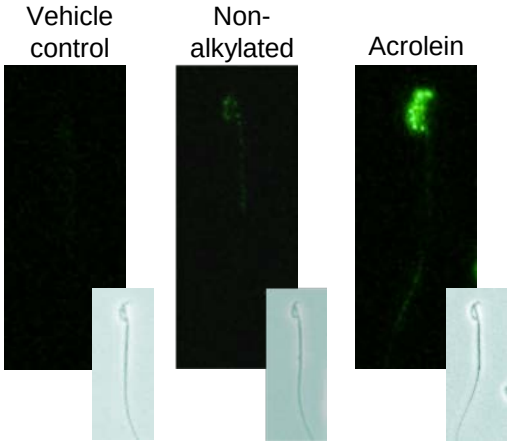


5

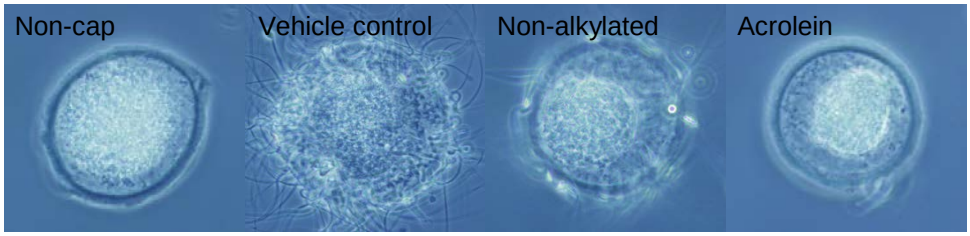
A

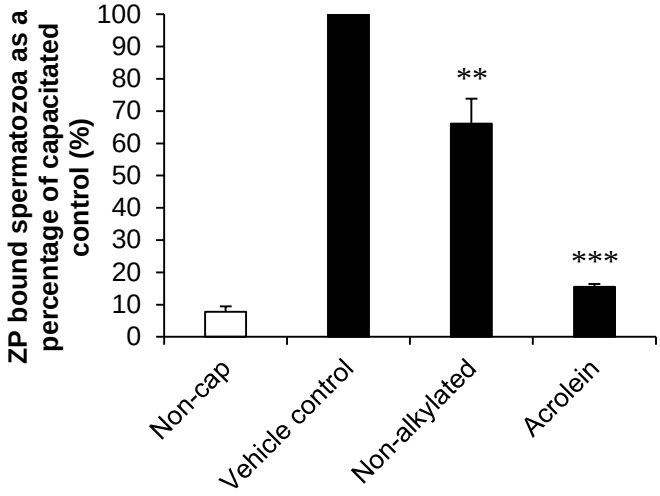

Motility
Progressive Motility
Motility (%)
100
90
80
70
60
50
40
30
20
10
0
Vehicle control
Non-alkylated
Acrolein

B

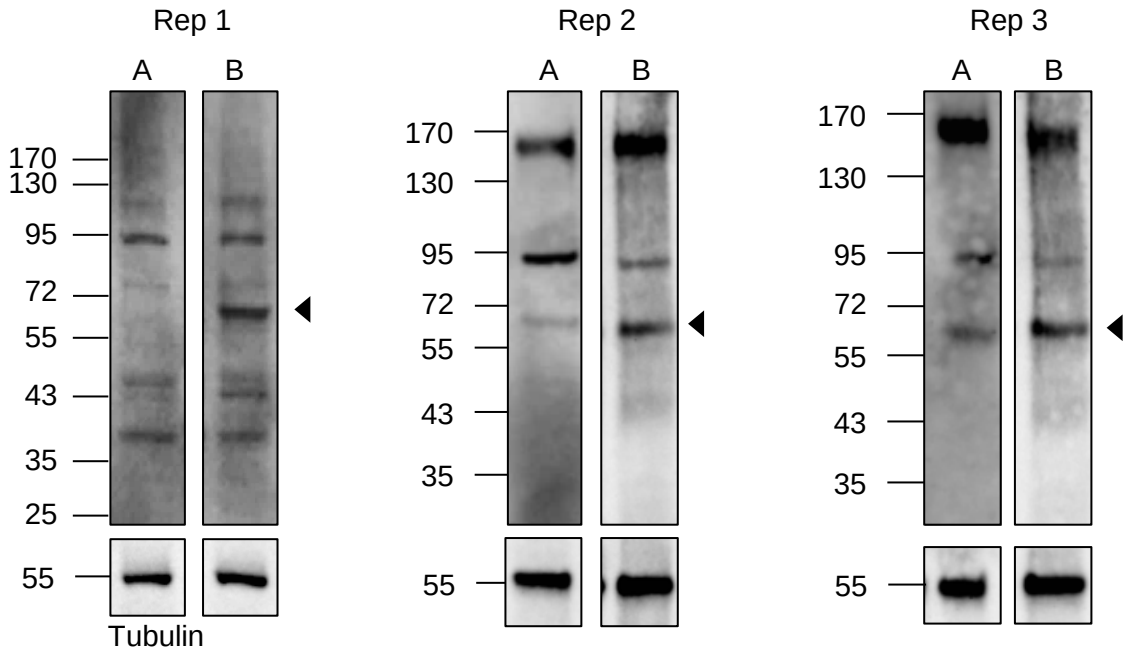

Vehicle control
Non-alkylated
Acrolein

C

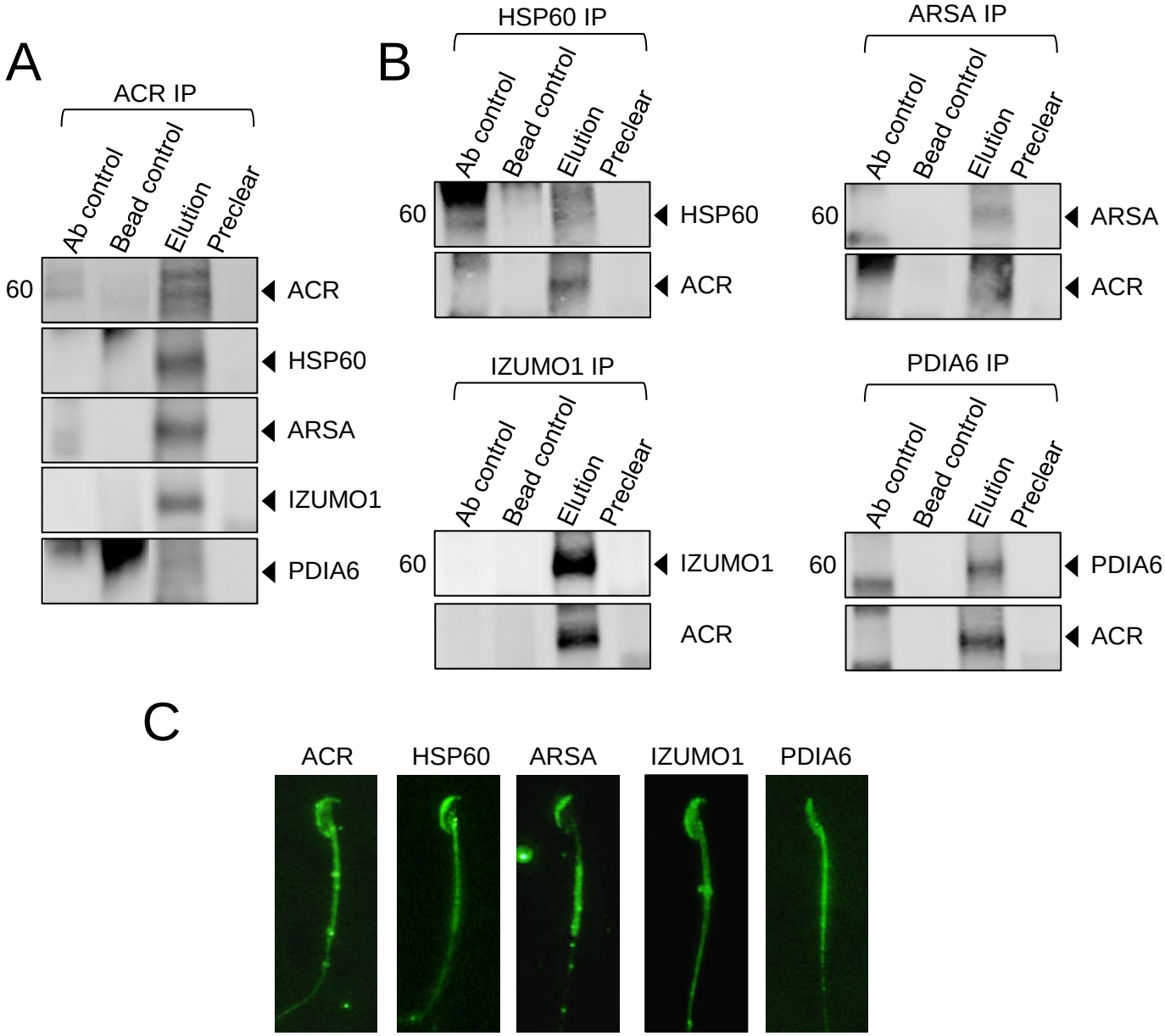

Non-cap
Vehicle control
Non-alkylated
Acrolein


ZP bound spermatozoa as a percentage of capacitated control (%)
100
90
80
70
60
50
40
30
20
10
0
**
***
Non-cap
Vehicle control
Non-alkylated
Acrolein

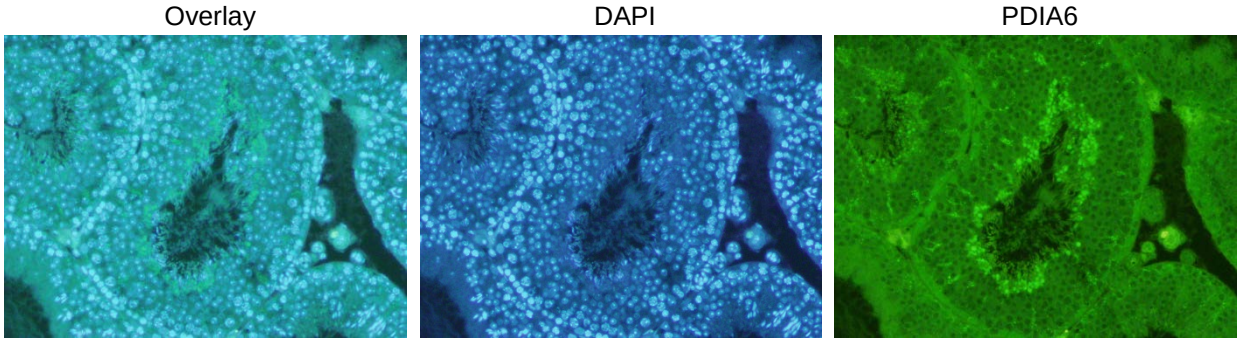
6


Rep 1
A
B
170
130
95
72
55
43
35
25
55
Tubulin
Rep 2
A
B
170
130
95
72
55
43
35
55
Rep 3
A
B
170
130
95
72
55
43
35
55

7


A
ACR IP
Ab control
Bead control
Elution
Preclear
60
ACR
HSP60
ARSA
IZUMO1
PDIA6
B
HSP60 IP
ARSA IP
IZUMO1 IP
PDIA6 IP
C
ACR
HSP60
ARSA
IZUMO1
PDIA6

8



CHAPTER 5

FINAL DISCUSSION

Chapter 5: Overview

The studies described in this thesis were designed to explore the biological principles necessary for the development of non-surgical methods of sterilisation for future application in free-ranging feral horses. Our findings in relation to mare folliculogenesis established that the signalling pathways that control follicle activation are indeed conserved in this species. These findings therefore offer the opportunity to manipulate follicular signalling pathways with local ovarian inhibitors or redox cycling quinones, in order to deplete the animal's reproductive potential. Importantly, these findings also permit the use of follicular signalling molecules such as PTEN and FOXO3A to be used as biomarkers to measure reproductive potential in the mare.

Following our findings in the mare, we next directed our attention to the stallion, whose gametes are more readily available and can offer contraceptive coverage for both sexes; making it a more ideal approach for integrating into feral horse management strategies. These studies exploited the knowledge that stallion spermatozoa are highly susceptible to oxidative stress owing to their unique reliance on OXPHOS. However, we extended previous observations by demonstrating that prominent lipid peroxidation products (ACR and 4HNE) generated as a consequence of this stress have the potential to covalently modify sperm proteins such as HSP90AA1 and ARSA. On the basis of previous research it is suggested that these represent key functional proteins with putative roles in the mediation of sperm-egg interactions. The modification of these proteins may thus be responsible, in part, for loss of zona pellucida binding competence observed in stallion spermatozoa exposed to exogenous ACR and 4HNE.

These findings have thus provided the impetus to perform a proof-of-concept immunisation and fertility trial in the mouse model. Such a trial was formulated on

the principle that covalent modification of proteins by electrophilic aldehydes can enhance their immunogenicity as reported in certain cases of autoimmunity. In these studies we discovered that immunisation with ACR-adducted sperm proteins resulted in fewer foetuses following an *in vivo* mating trial and successfully reduced zona pellucida binding competence *in vitro*, possibly due to the generation of antibodies against a number of covalently modified proteins implicated in gamete interaction and fusion. The following chapter provides a summary of the entire thesis, incorporating all of the aforementioned concepts together to achieve a long-term fertility control method for feral horses.

Chapter 5: Final Discussion

5.1 Introduction

Non-surgical methods of sterilisation, whereby after a single administration the animal becomes infertile, offers the most effective and sustainable means of population control that has a high likelihood of public acceptance. In Chapter 1, we discussed a variety of mechanisms that can be used to achieve this; however, prior to implementing these technologies it was first necessary to (i) gain insight into the anatomy and molecular signalling of the mare ovary, (ii) assess the impact of electrophilic aldehydes on stallion spermatozoa, and (iii) determine whether aldehyde-adducted sperm proteins trigger an immune response in a similar manner to that which occurs in autoimmune disease.

The means by which non-surgical sterilisation technologies are likely to be successful is via local activation of an oxidative stress cascade. Indeed, oxidative stress is one of the key hallmarks of infertility [1-9]; its aetiology stemming from aging and exposure to environmental- and xeno- toxicants. It is a self-perpetuating system, whereby a step-by-step transfer of electrons occurs in response to ROS, resulting in the oxidation of lipids to form highly reactive aldehydes, which readily bind to the nucleophilic centres of vulnerable proteins, perturbing their function [10-14]. Such covalent modification of proteins was observed in stallion (Chapter 3) and mouse (Chapter 4) spermatozoa following exogenous exposure to electrophilic aldehydes, which resulted in a significant reduction in sperm-zona pellucida binding competence. The disruptive impact of both exogenous electrophilic aldehydes has also been established in the oocytes of mice [11], and is therefore an area worthy of investigation in the mare.

5.2 Manipulation of local ovarian signalling pathways

Gaining insight into mare folliculogenesis is particularly useful for non-surgical sterilisation in the context of redox agent development, which when targeting the ovary, triggers a highly redox active response that stimulates uncontrolled primordial follicle activation [15-19]. In Chapter 2, we discovered that PI3K/AKT and JAK/STAT signalling molecules do indeed exist in the mare ovary, consistent with other species (human [20-23]; mouse [24-28]; rabbit [27]; bovine [29]). Therefore, it may be possible to manipulate follicular signalling in the mare using xenobiotics such as the redox cycling quinone, menadione (2-methyl-1,4 naphthoquinone), which generates local ROS within the ovary and culminates in premature ovarian failure in the mouse model [30]. An ovary-specific PTEN inhibitor could also allow for unregulated PI3K/AKT pathway activation exhausting the primordial follicle pool [19, 23, 28, 30, 31]. However, further research in follicular activation is needed, particularly in the mare, as it is likely that other ovarian signals play a role in follicular activation.

Indeed, research into microRNA (miRNA) expression in preovulatory follicles has shown a triad of miRNAs responsible for decreased expression of PTEN [32]. This is rather novel because PTEN has previously been linked only to the primordial follicle stage of folliculogenesis [33]. Thus it is possible that miRNAs are also playing a role in primordial follicle activation. Additionally, other signalling pathways have been reported in follicular activation, including WNT [34, 35] and more recently HIPPO [36]. For what is such a critical aspect of female reproductive physiology, it is likely that many different, potentially interconnected, signalling pathways are responsible for initial recruitment. Thus the use of a single inhibitor may not be sufficient to cause premature ovarian failure in the mare, and a cocktail of inhibitors from different pathways may be necessary. There is still much to be learnt about the

molecular mechanisms and genes involved in ovarian development, and how their expression can be manipulated for the purpose of fertility control.

Random phage-peptide display was previously discussed in Chapter 1 [37] as the technology offers exquisite species-specificity and is able to target proteins that exist on primordial germ cells that would otherwise be unidentifiable. As previously discussed, targeted exposure to redox cycling quinones via phage-peptides would potentially provide a suitable method of non-surgical sterilisation. This method could theoretically also be used to deliver electrophilic aldehydes to gametes, as these alkylating agents are particularly toxic to primordial germ cells within the ovary and testis [38]. While spermatogonial stem cell and Sertoli cell isolation was not attempted from the testis of the stallion in this thesis, isolation of primordial follicles from the horse ovary was attempted and proved difficult. Indeed, due to the large size of the horse ovary and its unusual anatomical and cartilaginous structure, the techniques commonly used to isolate primordial follicles from the neonatal mouse ovary were not sufficient for the horse ovary. This was further complicated by the difficulty in collecting uniform tissue from mares of the same age and health status. Ideally, primordial follicles should be sourced from foetal horse ovaries, as it is at this point *in utero* that the animal begins to undergo folliculogenesis and the number of primordial follicles in the ovary is at its highest. Thus significant impediments need to be overcome if this technology is to be used in the future for development of a non-surgical method of sterilisation for feral horses.

5.3 Auto- and iso- antigenicity of sperm proteins following electrophilic aldehyde adduction

Another opportunity for the development of a non-surgical sterilisation method which could be used in both sexes is via immunisation of aldehyde-adducted sperm

proteins. To assess the validity of this technology for feral horse fertility control, it was necessary to: (i) understand the mechanism of covalent modification of sperm proteins, (ii) under what conditions these modifications occurred and (iii) whether they were sufficient to raise the immunogenicity of a protein to such an extent that autoimmunity would result. In Chapter 3, we assessed the first two aims using stallion spermatozoa and identified proteins of functional significance targeted for adduction by both ACR and 4HNE.

In Chapter 4, the fertility of mice was impeded following immunisation with ACR-adducted sperm proteins. This is a promising finding; however, it is not currently established whether the reduction in fertility was the result of immunity established in females or males. Future research is to be conducted to address this and determine whether such a strategy could be offered for both males and females. We appreciate that this study was conducted in a small cohort of mice, and will need to be repeated in a larger group before subsequent trials in a Brumby population are performed.

In relating this work back to the stallion, both HSP90AA1 and ARSA have putative roles in the mediation of sperm-egg interactions, and given the similar protein profile identified in Chapter 4 between aldehyde adduction *in vitro* compared with the proteins that were targeted by the immune system *in vivo*, it is hypothesised that modification of HSP90AA1 and ARSA would also occur following immunisation with aldehyde-adducted sperm proteins in the horse. ARSA has been previously discussed in Chapter 4, targeted for aldehyde-adduction in the spermatozoa of many species both directly (horse; Chapter 3 [39], mouse; Chapter 4) and indirectly (human; [10]). For the development of a non-surgical sterilisation method via autoimmunisation, HSP90 is also a target worthy of comment as Pires, et al. [40] have previously shown that modification of HSP90 contains major epitopes that are targeted in women exhibiting spontaneous immunity against ovarian tissue.

Aldehyde adduction is thought to alter the structure of proteins such as HSP90 and ARSA that they become powerful antigens triggering the formation of antibodies that compromise sperm function and particularly sperm-zona pellucida recognition. In light of these considerations it would be reasonable to propose that immunisation with aldehyde-adducted sperm proteins in the horse would control fertility by disrupting the process of conception. Furthermore, due to the OXPHOS nature of stallion spermatozoa, which results is larger outputs of ROS, it is possible that immunisation with aldehyde-adducted sperm proteins, which is known to include enzymes involved in mitochondrial electron transport such as succinic acid dehydrogenase [14], could result in a more dramatic suppression of fertility than observed in the glycolysis-dependent mouse sperm model.

Covalent modification of sperm proteins of functional significance in sperm-egg interaction was the focus of both Chapters 3 and 4; however, there remains a paucity of research into the impact of electrophilic aldehydes on the MII oocyte in the mare. Extrapolation of data secured in mouse models suggests that exposure of the MII oocyte to electrophilic aldehydes could reduce fertility and embryonic development [41]. Acquiring mature oocytes from equine ovaries is certainly not as difficult a task as primordial follicle isolation, and should permit identification of proteins vulnerable to adduction in the oocyte following exposure to ACR and 4HNE.

A multitude of proteins are targeted following aldehyde adduction, and in Chapter 4 an immune response was generated against all targeted proteins. The development of future immunocontraceptives could well involve an immunisation strategy targeting a suite of sperm-egg interaction proteins, rather than a single entity. While the fertilisation process is indeed intricate, it is also known to be highly adaptive; allowing successful fertilisation to occur despite oxidative challenge and deletion of sperm-specific proteins. Furthermore, a more uniform vaccination

protocol necessary for commercial use may contain recombinant versions of the covalently modified proteins identified in this and future studies.

5.4 Detoxification and ATP generation as targets for adduction

In Chapter 3, a loss of motility in stallion spermatozoa occurred in response to exogenous exposure to ACR and 4HNE. This is a previously reported phenomenon in stallion [42] and human [12-14] spermatozoa, which occurs due to covalent modification of proteins responsible for detoxification, ATP generation and the motility apparatus itself [43]. Whilst proteins involved in sperm-egg interaction were the focus of our studies in Chapters 3 and 4, investigation of detoxification and ATP generation proteins, which are susceptible to aldehyde adduction would also be of merit, and would be useful in a contraceptive if the problem of cell and tissue specificity could be overcome. Certainly, ALDH2 was identified in stallion spermatozoa as an important mitochondrial detoxification mechanism acutely protecting the mitochondria [39, 42]. Similarly, in human spermatozoa, succinate dehydrogenase was vulnerable to adduction by 4HNE and resulted in motility loss [14]. Consistently, glucose-6-phosphate isomerase (GPI), was identified as a vulnerable site for adduction in both stallion and mouse spermatozoa, localised to the mitochondria, and has previously been used as a predictor of sperm quality [44]. Moving forward it will be useful to investigate proteins within the stallion sperm proteome [45] involved in ATP generation and detoxification as they too hold putative roles, albeit indirect, in the ability of spermatozoa to fertilise an egg.

5.5 Considerations for fertility control application in the field

Whilst not the key focus of this thesis, field application is an important consideration for future development of a non-surgical method of sterilisation to ensure new technologies are readily implemented. Current immunisation strategies developed during this thesis will need to be manipulated to allow successful application for free-ranging feral horses, as administration by injection or darting is largely infeasible. Implementation of a passive fertility control management strategy such as a salt lick that has restricted access to horses would be ideal [37]. Another feature of administration that will need to be addressed is marking animals that have received the fertility control agent. In a bait delivery mechanism this could be achieved via chalk marking during the animal's visit. Once repeated attendance occurs at the bait delivery site, the device could then be relocated to a new area. Appreciation of the number of animals in an area in which fertility control intervention is to be implemented will also be crucial in ensuring appropriate coverage. Ideally, 90% bait coverage would be a successful outcome for feral horse management; however, management strategies may need to consider maintaining a fertile population to ensure the iconic Brumby is not lost and stakeholder and public needs are met. Nevertheless, it is unlikely that complete eradication will be achieved.

5.6 Concluding Remarks

The role of oxidative stress in infertility is well established, but what causes the onset of oxidative stress, and each milestone that occurs from this onset to subsequent fertility loss is less clear. This formed an important focus of our research, with the potential to use oxidative stress as a tool in our armoury for non-

surgical sterilisation of feral horses. In conclusion of this thesis, our findings suggest that signalling pathways necessary for primordial follicle activation, PI3K/AKT and JAK/STAT, are indeed conserved in the mare ovary, and future research can now focus on a non-surgical method of sterilisation that manipulates the rate of initial recruitment in the ovary. Secondly, analysis of the impact of electrophilic aldehydes on stallion spermatozoa has revealed proteins vulnerable to adduction, HSP90AA1 and ARSA by ACR and 4HNE, respectively. Immunisation with aldehyde-adducted sperm proteins in the mouse appears sufficient to impede fertility, following covalent modification of a variety of proteins involved in sperm-egg interaction (HSP60, ARSA, IZUMO1, PDIAs). Whilst evidence in the mouse model is not intended to be a definitive investigation into immunisation with covalently modified sperm proteins, our findings offer tangible hope for future contraceptive development in this area.

References

1. Agarwal A, Gupta S, Sharma R. Oxidative Stress and Its Implications in Female Infertility–a Clinician's Perspective. Reproductive Biomedicine Online 2005; 11:641-650.
2. Aitken RJ, Baker MA. Oxidative Stress, Sperm Survival and Fertility Control. Mol Cell Endocrinol 2006; 250:66-69.
3. Aitken RJ, Baker MA. Oxidative Stress, Spermatozoa and Leukocytic Infiltration: Relationships Forged by the Opposing Forces of Microbial Invasion and the Search for Perfection. J Reprod Immunol 2013; 100:11-19.
4. Aitken RJ, Baker MA, Nixon B. Are Sperm Capacitation and Apoptosis the Opposite Ends of a Continuum Driven by Oxidative Stress? Asian Journal of Andrology 2015.

5. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and Consequences of Oxidative Stress in Spermatozoa. Reprod Fertil Dev 2015; 28:1-10.
6. Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative Stress and Male Reproductive Health. Asian Journal of Andrology 2014; 16:31.
7. Gharagozloo P, Aitken RJ. The Role of Sperm Oxidative Stress in Male Infertility and the Significance of Oral Antioxidant Therapy. Hum Reprod 2011; 26:1628-1640.
8. Lord T, Aitken RJ. Oxidative Stress and Ageing of the Post-Ovulatory Oocyte. Reproduction 2013; 146:R217-R227.
9. Ruder EH, Hartman TJ, Blumberg J, Goldman MB. Oxidative Stress and Antioxidants: Exposure and Impact on Female Fertility. Hum Reprod Update 2008; 14:345-357.
10. Bromfield EG, Aitken RJ, Anderson AL, McLaughlin EA, Nixon B. The Impact of Oxidative Stress on Chaperone-Mediated Human Sperm–Egg Interaction. Hum Reprod 2015:dev214.
11. Lord T, Martin JH, Aitken RJ. Accumulation of Electrophilic Aldehydes During Post-Ovulatory Ageing of Mouse Oocytes Causes Reduced Fertility, Oxidative Stress, and Apoptosis. Biol Reprod 2014:biolreprod. 114.122820.
12. Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, Aitken RJ. Oxidative Stress and Human Spermatozoa: Diagnostic and Functional Significance of Aldehydes Generated as a Result of Lipid Peroxidation. Mol Hum Reprod 2015; 21:502-515.
13. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm Motility Is Lost in Vitro as a Consequence of Mitochondrial Free Radical Production and the Generation of Electrophilic Aldehydes but Can

Be Significantly Rescued by the Presence of Nucleophilic Thiols. Biol Reprod 2012; 87:110.

14. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic Aldehydes Generated by Sperm Metabolism Activate Mitochondrial Reactive Oxygen Species Generation and Apoptosis by Targeting Succinate Dehydrogenase. J Biol Chem 2012; 287:33048-33060.
15. Sobinoff A, Pye V, Nixon B, Roman S, McLaughlin E. Xenobiotics: Influence on Ovarian Follicular Development. Reprod Fertil Dev 2009; 21:71-71.
16. Sobinoff A, Pye V, Nixon B, Roman S, McLaughlin E. Jumping the Gun: Smoking Constituent Bap Causes Premature Primordial Follicle Activation and Impairs Oocyte Fusibility through Oxidative Stress. Toxicol Appl Pharmacol 2012; 260:70-80.
17. Sobinoff AP, Nixon B, Roman SD, McLaughlin EA. Staying Alive: Pi3k Pathway Promotes Primordial Follicle Activation and Survival in Response to 3mc-Induced Ovotoxicity. Toxicol Sci 2012; 128:258-271.
18. Sobinoff AP, Pye V, Nixon B, Roman SD, McLaughlin EA. Adding Insult to Injury: Effects of Xenobiotic-Induced Preantral Ovotoxicity on Ovarian Development and Oocyte Fusibility. Toxicol Sci 2010; 118:653-666.
19. Schmid AC, Byrne RD, Vilar R, Woscholski R. Bisperoxovanadium Compounds Are Potent Pten Inhibitors. FEBS Lett 2004; 566:35-38.
20. Pastuschek J, Poetzsch J, Morales-Prieto DM, Schleußner E, Markert UR, Georgiev G. Stimulation of the Jak/Stat Pathway by Lif and Osm in the Human Granulosa Cell Line Cov434. J Reprod Immunol 2015; 108:48-55.
21. Tuck AR, Robker RL, Norman RJ, Tilley WD, Hickey TE. Expression and Localisation of C-Kit and Kitl in the Adult Human Ovary. Journal of Ovarian Research 2015; 8:31.

22. Huntriss J, Lu J, Hemmings K, Bayne R, Anderson R, Rutherford A, Balen A, Elder K, Picton HM. Isolation and Expression of the Human Gametocyte-Specific Factor 1 Gene (Gtsf1) in Fetal Ovary, Oocytes, and Preimplantation Embryos. J Assist Reprod Genet 2016.
23. McLaughlin M, Kinnell HL, Anderson RA, Telfer EE. Inhibition of Phosphatase and Tensin Homologue (Pten) in Human Ovary in Vitro Results in Increased Activation of Primordial Follicles but Compromises Development of Growing Follicles. Mol Hum Reprod 2014; 20:736-744.
24. Keightley RA, Nixon B, Roman SD, Russell DL, Robker RL, McLaughlin EA. Regulating Murine Follicle Development by Stimulation of the Jak2/Stat3 Signal Transduction Pathway. Reprod Fertil Dev 2009; 21:108-108.
25. Sutherland J, Keightley R, Nixon B, Roman SD, Robker RL, Russell DL, McLaughlin E. Suppressor of Cytokine Signaling 4 (Socs4): Moderator of Ovarian Primordial Follicle Activation. J Cell Physiol 2012; 227:1188-1198.
26. Zhang H, Risal S, Gorre N, Busayavalasa K, Li X, Shen Y, Bosbach B, Brannstrom M, Liu K. Somatic Cells Initiate Primordial Follicle Activation and Govern the Development of Dormant Oocytes in Mice. Curr Biol 2014; 24:2501-2508.
27. Hutt KJ, McLaughlin EA, Holland MK. Kit/Kit Ligand in Mammalian Oogenesis and Folliculogenesis: Roles in Rabbit and Murine Ovarian Follicle Activation and Oocyte Growth. Biol Reprod 2006; 75:421-433.
28. Jagarlamudi K, Liu L, Adhikari D, Reddy P, Idahl A, Ottander U, Lundin E, Liu K. Oocyte-Specific Deletion of Pten in Mice Reveals a Stage-Specific Function of Pten/Pi3k Signaling in Oocytes in Controlling Follicular Activation. PLoS ONE 2009; 4:e6186.
29. Ndiaye K, Castonguay A, Benoit G, Silversides DW, Lussier JG. Differential Regulation of Janus Kinase 3 (Jak3) in Bovine Preovulatory Follicles and

Identification of Jak3 Interacting Proteins in Granulosa Cells. J Ovarian Res 2016; 9:71.

30. Yoshikawa K, Nigorikawa K, Tsukamoto M, Tamura N, Hazeki K, Hazeki O. Inhibition of Pten and Activation of Akt by Menadione. Biochimica et Biophysica Acta (BBA) - General Subjects 2007; 1770:687-693.
31. Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible Inactivation of the Tumor Suppressor Pten by H2o2. J Biol Chem 2002; 277:20336-20342.
32. Schauer S, Sontakke S, Watson E, Esteves C, Donadeu FX. Involvement of Mirnas in Equine Follicle Development. Reproduction 2013; 146:273-282.
33. Adhikari D, Gorre N, Risal S, Zhao Z, Zhang H, Shen Y, Liu K. The Safe Use of a Pten Inhibitor for the Activation of Dormant Mouse Primordial Follicles and Generation of Fertilizable Eggs. PLoS ONE 2012; 7.
34. Boyer A, Goff AK, Boerboom D. Wnt Signaling in Ovarian Follicle Biology and Tumorigenesis. Trends in Endocrinology & Metabolism 2010; 21:25-32.
35. Jääskeläinen M, Prunskaite-Hyyryläinen R, Naillat F, Parviainen H, Anttonen M, Heikinheimo M, Liakka A, Ola R, Vainio S, Vaskivuo TE, Tapanainen JS. Wnt4 Is Expressed in Human Fetal and Adult Ovaries and Its Signaling Contributes to Ovarian Cell Survival. Mol Cell Endocrinol 2010; 317:106-111.
36. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, Ho CH, Kawamura N, Tamura M, Hashimoto S, Sugishita Y, Morimoto Y, et al. Hippo Signaling Disruption and Akt Stimulation of Ovarian Follicles for Infertility Treatment. PNAS 2013; 110:17474-17479.
37. Hall SE, Nixon B, Aitken RJ. Non-Surgical Sterilisation Methods May Offer a Sustainable Solution to Feral Horse (Equus Caballus) Overpopulation. Reprod Fertil Dev 2016:10.1071/RD16200.

38. Gadducci A, Cosio S, Genazzani AR. Ovarian Function and Childbearing Issues in Breast Cancer Survivors. Gynecol Endocrinol 2007; 23:625-631.

39. Hall SE, Aitken RJ, Nixon B, Smith ND, Gibb Z. Electrophilic Aldehyde Products of Lipid Peroxidation Selectively Adduct to Heat Shock Protein 90 and Arylsulfatase a in Stallion Spermatozoa. Biol Reprod 2017:bio145292.

40. Pires ES, Choudhury AK, Idicula-Thomas S, Khole VV. Anti-Hsp90 Autoantibodies in Sera of Infertile Women Identify a Dominant, Conserved Epitope Ep6 (380-389) of Hsp90 Beta Protein. Reprod Biol Endocrinol 2011; 9:16.

41. Lord T, Martin J, Aitken R. Accumulation of 4-Hydroxynonenal During Post-Ovulatory Ageing of Mouse Oocytes Causes Reduced Fertility, Oxidative Stress and Apoptosis. Fertil Steril 2014; 102:e330.

42. Gibb Z, Lambourne SR, Curry BJ, Hall SE, Aitken RJ. Aldehyde Dehydrogenase Plays a Pivotal Role in the Maintenance of Stallion Sperm Motility. Biol Reprod 2016; 94:133.

43. Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J, Gordon C. Defining the Mechanisms by Which the Reactive Oxygen Species by-Product, 4-Hydroxynonenal, Affects Human Sperm Cell Function. Biol Reprod 2015:biolreprod. 114.126680.

44. Jiang X-p, Wang S-q, Wang W, Xu Y, Xu Z, Tang J-y, Sun H-y, Wang Z-j, Zhang W. Enolase1 (Eno1) and Glucose-6-Phosphate Isomerase (Gpi) Are Good Markers to Predict Human Sperm Freezability. Cryobiology 2015; 71:141-145.

45. Swegen A, Curry BJ, Gibb Z, Lambourne SR, Smith ND, Aitken RJ. Investigation of the Stallion Sperm Proteome by Mass Spectrometry. Reproduction 2015; 149:235-244.