

RESEARCH ARTICLE

# Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (*Equus caballus*): Limitations and side effects

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## Abstract

Wildlife and humans are increasingly competing for resources worldwide, and a diverse, innovative, and effective set of management tools is needed. Controlling abundance of wild-life species that are simultaneously protected, abundant, competitive for resources, and in conflict with some stakeholders but beloved by others, is a daunting challenge. Free-ranging horses (*Equus caballus*) present such a conundrum and managers struggle for effective tools for regulating their abundance. Controlling reproduction of female horses presents a potential alternative. During 2009–2017, we determined the long-term effectiveness of GnRH vaccine (GonaCon-Equine) both as a single immunization and subsequent reimmunization on reproduction and side effects in free-ranging horses. At a scheduled management roundup in 2009, we randomly assigned 57 adult mares to either a GonaCon-Equine treatment group ( $n = 29$ ) or a saline control group ( $n = 28$ ). In a second roundup in 2013, we administered a booster vaccination to these same mares. We used annual ground observations to estimate foaling proportions, social behaviors, body condition, and injection site reactions. We found this vaccine to be safe for pregnant females and neonates, with no overt deleterious behavioral side effects during the breeding season. The proportion of treated mares that foaled following a single vaccination was lower than that for control mares for the second ( $P = 0.03$ ) and third ( $P = 0.08$ ) post-treatment foaling seasons but was similar ( $P = 0.67$ ) to untreated mares for the fourth season, demonstrating reversibility of the primary vaccine treatment. After two vaccinations, however, the proportion of females giving birth was lower ( $P < 0.001$ ) than that for control mares for three consecutive years and ranged from 0.0–0.16. The only detectable adverse side effect of vaccination was intramuscular

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swelling at the vaccination site. Regardless of vaccine treatment (primary/secondary), approximately 62% (34/55) of immunized mares revealed a visible reaction at the vaccine injection site. However, none of these mares displayed any evidence of lameness, altered gait or abnormal range of movement throughout the 8 years they were observed in this study. Our research suggests that practical application of this vaccine in feral horses will require an initial inoculation that may provide only modest suppression of fertility followed by reimmunization that together could result in greater reduction in population growth rates over time.

## Introduction

Anthropogenic disturbance of landscapes and natural resources is pervasive across much of the earth, resulting in increased conflict between humans and wildlife and a need for effective resource management [1]. Humans indeed have tried to control animal abundance in some capacity for over 13,000 years [2]. Regulating abundance of wild animals using fertility control or contraception is a relatively new development, emerging only 50 years ago [3]. Such tools are appealing to wildlife managers and stakeholders because they present a non-lethal solution for regulating abundance when species pose a risk to human interests and safety, and when wildlife densities are high enough to disrupt ecosystem function [4,5].

Feral horses (*Equus caballus*) present perhaps one of the most unique wildlife management problems worldwide. Humans have spent centuries propagating and dispersing domestic horses to every continent except Antarctica over the last several centuries, only to have inadvertently created expansive feral populations that now compete with humans, wildlife, and domestic animals for resources [6]. The unique relationship between humans and horses has resulted in a precarious dichotomy, with the struggle for relief from conflict and resource competition challenged by a mutualistic societal view where feral horses are perceived as part of our social environment. This struggle is elevated in the United States, where federal law (*P. L. 92–195*, as amended) provides protection for feral horses and burros (*Equus asinus*) on large expanses of public land, and establishes guidance for their management as a wildland species [7].

Current methods of population control for free-ranging horses in the U.S. involve periodic removals and adoption or sale of surplus animals, or maintaining excess animals in long-term holding facilities which are expensive, resource intensive, and unsustainable [8]. Clearly, more efficient, cost effective, and humane approaches to reducing feral horse densities on public lands are needed. Controlling the fertility of female horses offers a potential complementary or alternative strategy for limiting the growth of some populations [9].

A promising immunological approach to contraception in feral horses and other wild ungulate species involves immunization against gonadotropin-releasing hormone (GnRH), a small neuropeptide that performs an obligatory role in mammalian reproduction [10]. When conjugated to a highly immunogenic carrier protein and combined with a potent adjuvant, GnRH vaccination actively stimulates a persistent immune response resulting in prolonged antibody production against endogenous GnRH. These antibodies induce transient infertility by binding to GnRH, thus preventing attachment to receptors on pituitary gonadotropes, suppression of gonadotropin release, and ultimately ovulation in females [11, 12]. As anti-GnRH antibodies decline over time, the availability of endogenous GnRH increases and treated animals generally regain normal fertility [13–17].

The GnRH-based contraceptive agent known as GonaCon-Equine (National Wildlife Research Center, Fort Collins, CO, USA; [18] is registered by the United States Environmental Protection Agency as a restricted-use pesticide for contraception of adult female feral horses and burros. A single immunization with this or earlier versions of this vaccine (more generally referred to as GonaCon) have been shown to induce extended infertility ( $\geq 2$  yr) in numerous wild ungulate species including captive and free-ranging elk (*Cervus elaphus*) [15–17] white-tailed deer (*Odocoileus virginianus*) [18–20], bison (*Bison bison* [21]), and feral horses [22–24]. However, multiple years of infertility are only experienced in a fraction of vaccinated animals. In free-ranging elk, for example, there was approximately a 90% treatment effect the first year after vaccination but this declined to 50% by the second year; with no measurable effect by year three [16]. Similar declines in effectiveness have been reported for captive feral horses treated with the same vaccine [22].

Booster vaccinations generally result in a more profound and longer-lasting antibody production due to the anamnestic (cell memory) response [25]. Traditional veterinary vaccinology suggests that non-replicating vaccines most often require two initial doses 2–6 weeks apart followed by booster vaccinations every 1–3 years [26]. Repeat immunizations using a variety of GnRH vaccines in domestic horses improves contraceptive efficacy and suppress behavioral and physiological estrus [27–29]. However, these GnRH vaccines differ from GonaCon-Equine in that they incorporate different protein carrier molecules and adjuvants, and are formulated for short duration ( $< 1$  yr.) effectiveness. They are also administered on a more traditional vaccination schedule with a primary set of immunizations followed by periodic boosters.

Other forms of wildlife fertility control vaccines have adopted comparable initial and booster recommendations [30–32]. However, this intensive vaccination schedule places significant logistical barriers on application in free-ranging animals. GonaCon vaccine is formulated with highly immunostimulating mycobacteria as a component of the adjuvant. This may prolong the initial and subsequent booster vaccination windows for optimum efficacy as initial antibody concentrations are maximal 2–12 months post-primary vaccination [15]. GonaCon vaccine is one of the rare exceptions among animal vaccines in that the formulation initiates high antibody titers that remain elevated in some individuals after a single-injection; however, little research has been conducted to evaluate booster doses of this vaccine in any free-ranging wild ungulate [17, 24] or domestic species [33]. While a single immunization against GnRH may be preferred from a practical perspective, there may be a more optimal vaccination schedule that balances the need for minimizing animal handling or contact while maximizing vaccine effectiveness. Thus, it's imperative to investigate the safety and long-term effectiveness of repeat vaccination and to evaluate its potential to limit fertility in this long-lived and perennially pregnant species.

In female wild ungulates, adverse side effects following a single immunization against GnRH appear to be minimal. Evaluation of biological side effects has been reported for numerous wild ungulate species including white-tailed deer [13, 34], elk [15, 16, 35], feral pigs [36], bison [21], and free-ranging horses [17, 24]. A summary of results from these investigations indicate that GonaCon is reversible, safe for use in pregnant females, does not significantly change social behaviors [37] or negatively affect neonatal development, survival, or maturation [15, 35]. No adverse effects of vaccination have been shown to be related to general health, body condition, blood chemistry parameters, or hematology of treated animals. The most apparent pathological side effect has been the development and persistence of non-debilitating granulomatous and often purulent inflammation at the site of injection. In all studies, where post-mortem examinations have been conducted, injection-site lesions were pervasive, but in some species, such as white-tailed deer and elk, they were not apparent antemortem. Likewise,

in cases where injection-site reactions have been documented, no clinical evidence of lameness, impaired mobility, or depression, have been reported [13, 15–17, 21, 24, 34, 35].

While documentation of contraceptive efficacy and side effects of GonaCon have been described for a variety of wild ungulates, similar evidence for feral horses is limited. To our knowledge, only two long-term ( $\geq 3$  years) empirical investigations have been conducted using GonaCon-Equine. These include a clinical trial with captive feral mares [22] and the other with free-ranging mares in a natural environment [23]. In the study with free-ranging horses, vaccination significantly reduced foaling rates of treated females, however, effectiveness was inconsistent over time and was substantially lower than that reported for captive feral mares treated with the same vaccine [22]. Furthermore, neither of these studies integrated revaccination as a strategy to increase vaccine efficacy. Lastly, these inquiries provide little quantitative evidence of the reversibility of the effects of this vaccine, the presence or absence of adverse side effects related to inoculation of pregnant mares, and neither examined the potential for increased side effects with reimmunization.

Knowledge of the effects of GonaCon-Equine on equid fetal health, neonatal survival, and body condition is largely anecdotal, whereas injections site reactions to booster immunization and the efficacy of revaccination are limited to two investigations [24, 33]. Clearly, additional research is needed to further define the long-term therapeutic effectiveness and contraindications of this potential technology before resource managers can make informed decisions regarding its practical application for stabilizing the growth rate of free-ranging feral horse populations.

Consequently, the fundamental objectives of this investigation were: 1) to determine the duration, effectiveness, and reversibility of both a single immunization and subsequent reimmunization against GnRH in suppressing reproductive rates of free-ranging mares in a natural environment, 2) to determine the safety and adverse side effects (if any) in free-ranging mares including assessment of general health, body condition, effects on current pregnancy, injection site reactions, and neonatal health and survival and, 3) to compare the effects of a single vaccination against GnRH on time budgets and social behaviors [37] to similar behaviors following reimmunization. Based on evidence from prior studies with feral horses and other wildlife species, we predicted ( $H_{1.}$ ) that a single vaccination against GnRH would suppress fertility for multiple years with decreasing effectiveness over time but would not result in permanent infertility. Furthermore, we surmised ( $H_{2.}$ ) that the anamnestic immune response to revaccination would be more effective and longer lasting in suppressing fertility than the initial immunization alone. Moreover, we reasoned ( $H_{3.}$ ) that except for localized inflammatory reactions at the injection site, we would not observe other adverse side effects (i.e. lameness, detrimental effects on existing pregnancy, neonatal health and survival, body condition, behavioral changes). Apart from determination of return to normal fertility of treated mares, these objectives and hypotheses were addressed and accomplished in this investigation.

## Materials and methods

### Study area

We conducted this research in the South Unit of Theodore Roosevelt National Park (THRO), USA ( $45^{\circ} 55'N/103^{\circ} 31'W$ ). This unit is located near the town of Medora in southwestern North Dakota and encompasses approximately 19,000 ha of native vegetation. The landscape is topographically diverse and consists of eroded badlands with gullies and ravines separated by relatively large upland plateaus and small erosion-resistant buttes capped by scoria. Elevation ranges from 683 m to 870 m. Its continental climate is characterized by short, arid summers (mean temperature  $21^{\circ}C$ ) and long, cold winters (mean temperature  $-12^{\circ}C$ ) [38].

Precipitation is irregular in amount and distribution with a long-term annual mean of 38 cm with most of this falling as rain showers from April to June [39].

Vegetation is primarily mixed-grass prairie dominated by needle-and-thread grass (*Hesperostipa comata*), western wheatgrass (*Pascopyrum smithii*), threadleaf sedge (*Carex filifolia*), blue gramma (*Boutelous gracilis*), and little blue-stem (*Schizachyrium scoparium*). Cottonwood (*Populus deltoides*) gallery forests occur along perennial water courses while hardwood stands of green ash (*Fraxinus pennsylvanica*) and chokecherry (*Prunus virginiana*) dominate the upland drainages. Dense stands of Rocky Mountain juniper (*Juniper scopulorum*) are common on steep north-facing slopes [40].

Besides feral horses, sympatric wild ungulate species include bison, elk, mule deer (*Odocoileus hemionus*), white-tailed deer, and pronghorn (*Antilocapra americana*). Horses and bison are confined to the South Unit of the Park by a 1.8–2.4 m woven-wire boundary fence. Currently, horse numbers are controlled through periodic live capture and removal of select individuals. Free-ranging horses at THRO are classified by the National Park Service (NPS) as “feral livestock” and managed as a “historical demonstration herd”. The most recent estimate of population size (2017) is 150–175 horses and the Park has set a management goal for this herd at approximately 50–90 animals.

The social structure of this population consists of 14–16 social groups (bands) that include a single dominant stallion, subdominant stallions, and 1–5 adult mares, yearlings, and foals of both sexes. Males greater than 1 year of age that have not acquired a band are usually found in ephemeral bachelor groups of 3–6 individuals. These bands are non-territorial and are spatially distributed across the South Unit primarily east of the Little Missouri River. All horses are known by unique coloration and markings and have been previously identified and assigned individual identifiers by managers. Photographs of each animal from birth to adulthood assist in the identification of individuals. Age, reproductive history, and genealogy data for each animal has been maintained since 1993.

In spring/summer 2009, we collected pre-treatment data on all mares and bands within THRO. The purpose of this effort was: 1) to determine the sample size and sampling intensity required to achieve acceptable statistical power ( $\geq 80\%$ ) to detect fixed differences ( $\geq 50\%$ ) in foaling proportions of experimental groups, 2) to assess unknown logistical limitations of locating and identifying specific study mares within bands of horses, and 3) to train field technicians to observation protocols, and collect pre-treatment time budget and social behavioral data.

## Experimental animals and treatments

**Primary vaccination (2009–2013).** During a scheduled management roundup at THRO (18–23 October 2009), 160 horses were guided by helicopter into permanent corrals and handling facilities. An attempt was made to capture the entire population to maximize sample sizes for this research project and to remove excess horses to meet desired herd management objectives. A total of 57 adult mares (2–17 years of age) and associated foals, and band stallions, were captured, identified, treated, and retained in the Park for this experiment. Using a randomized complete block design, we established two experimental groups consisting of a Gona-Con-Equine treatment group ( $n = 29$ ) and a saline control group ( $n = 28$ ). Mares were paired (blocked) based on age and pregnancy status such that animals within a block were as similar as possible. Within each block, individual mares were then randomly assigned to either a control or treatment group.

Equine veterinarians and a reproductive specialist, blinded to treatment status, assessed the general health, body condition, pregnancy status, and approximate gestational stage of each

mare. We determined pregnancy status and gestational age by transrectal palpation and ultrasonography of the reproductive tract [41]. We collected whole blood (up to 50 mL) via jugular venipuncture (BD Vacutainer SST; Becton Dickinson and Co., Franklin Lakes, NJ) then centrifuged these samples at the capture site, and temporarily stored serum in cryovials at  $-20^{\circ}\text{C}$ . We later transferred frozen serum on dry ice to Fort Collins, Colorado, where it was stored at  $-80^{\circ}\text{C}$ . We also assessed serum for exposure to common pathogens known to cause abortions in horses (e.g. equine herpesvirus-1, equine infectious anemia, equine viral arteritis and contagious equine metritis) that could confound the interpretation of treatment-induced infertility [42].

We applied treatments while mares were restrained in a squeeze chute. Females in the treatment group received an intramuscular injection in the lower left gluteal musculature, by hand-held syringe (18-gauge, 3.8 cm needle) containing GonaCon-Equine (2.0 mg GnRH conjugate + adjuvant; 2.0 mL). The vaccine contained multiple synthetic copies of GnRH coupled to a large immunogenic carrier protein (Blue Carrier; Biosonda, Santiago, Chile) that was combined with a water-in-oil adjuvant containing killed *Mycobacterium avium* ssp. *avium* (AdjuVac, National Wildlife Research Center) [18]. Mares in the control group were injected in a similar manner, with an equal volume of physiologic saline solution (0.9% NaCl; 2.0 mL). We chose to inject the vaccine into the gluteus muscle (~ 15 cm distal to the point of the hip) rather than the neck because of greater safety for hand-injection, enhanced detection of potential injection site reactions under field conditions, and the preferred location for potential remote dart delivery of the vaccine.

**Secondary vaccination (2013–2017).** Four years later, during 23–25 September 2013, we similarly rounded up the entire THRO horse population and moved and handled them through existing corrals and chute systems to remove excess animals from the Park. Given this unique opportunity and endorsement from the Park, we retained all available mares previously immunized and control mares, retreated them, assessed pregnancy status, and determined body condition using techniques identical to those applied at the 2009 roundup. Two mares in the control group and 4 mares in the treatment group died between 2009–2013 and therefore, were not available for this experiment. We attributed these mortalities to malnutrition, dystocia, broken appendage, and unknown causes not related to treatments. The one exception in our 2013 protocol was that we injected the booster vaccination into the opposite (right) hip from where the primary (left hip) vaccination was previously administered. This provided the opportunity to simultaneously evaluate injection site reactions related to both immunizations. Treatment mares again received 2.0 mL GonaCon-Equine and control mares 2.0 mL saline.

## Field measurements

Using 2–3 trained technicians and occasional equally trained volunteers, we conducted field measurements and observations consistently from year to year. Prior to field observations, technicians were provided with photographic images of individual horses and required to recognize them by band association, natural markings, and pelage coloration. They were also trained or had previous experience in identifying prepartum characteristics of pregnancy (e.g., enlarged abdomen, mammary gland development, waxing teats, behavior, etc.), as well as, body condition scoring, and the appearance and classification of injection site reactions to the vaccine. We collected all data from ground surveys (foot, vehicle, horseback) using binoculars and spotting scopes. Although technicians were unaware of treatment assignments of individual mares, the presence of injection site reactions in several GonaCon-treated mares could have revealed their treatment designation.

**Reproduction.** We predicted that pregnant females inoculated with GnRH at the fall gathers of 2009 and 2013 would give birth to a healthy foal the following spring (2010 or 2014) and presumably be infertile during subsequent breeding seasons. Thus, the effects of the primary or booster vaccinations on reproduction (foaling proportions) would not be observed until the 2011 and 2015 foaling seasons, respectively. These are the first breeding seasons that a treatment or retreatment effect on mare fertility could be detected when using foaling observations to assess successful contraception by the vaccine.

We determined the effectiveness, duration of effects, and reversibility of the primary and booster vaccinations on reproduction by comparing foaling proportions of treated and control mares during 1 March to 31 December 2009–2017. We chose to use the term vaccine “effectiveness” rather than “efficacy” because it more realistically represents how GonaCon-Equine affects fertility under more natural field conditions compared to a controlled clinical trial [43, 44]. We defined vaccine effectiveness (VE) as the proportional reduction in annual foaling ( $F$  = number of mares with a foal/ total number of mares in a treatment group) between control and treated mares. Vaccine effectiveness is equivalent to relative risk reduction (RRR) in medical statistics and was calculated from the risk ratio ( $RR = \frac{F_{Trt}}{F_{Con}}$ ) where  $F_{Con}$  = foaling proportion of the control mares, and  $F_{Trt}$  = the foaling proportion of the treated mares. Risk ratio was calculated using the *fmsb* package in program R [45–47] and we then solved for VE as follows:

$$VE = \frac{F_{Con} - F_{Trt}}{F_{Con}} = 1 - \frac{F_{Trt}}{F_{Con}} = 1 - RR$$

Each year of the study, we estimated annual foaling proportions by locating all bands to identify individual mares and determine the presence or absence of foals. During the intensive sampling period (1 March–1 August), we attempted to observe 95% or greater of all experimental mares and foals (when present) at least weekly and 100% of them every two weeks, then opportunistically until 31 December. We did not attempt to assess contraceptive effect based on visual characteristics of pregnancy but did use these criteria to prioritize weekly observations of individual mares. Instead, we defined foaling as a parturition event or neonatal foal by side, as detected by direct observation. We matched foals with dams through observations of nursing and repeated close association during feeding, bedding, and traveling [48, 49]. We collected neonatal data at first sighting of a foal and estimated date of birth by observing the foal’s level of activity, presence of an umbilicus, and elapsed time since the dam was last observed pregnant [50]. We photographed and estimated the age of each new foal when first observed, recorded its sex, general health (vigorous, average, poor), markings, and band association, and gave it a unique identifier; then entered these observations into a herd database. Finally, we assessed the utility of using foaling proportions as a proxy for pregnancy proportions by comparing pregnancy proportions determined at the time of each gather in 2009 and 2013 to foaling proportions observed in 2010 and 2014.

## Side effects

**Behavioral.** We repeated three behavioral measurements with the same treatment groups of mares that were previously conducted during an earlier phase of this project [37]. We proposed that, if greater contraceptive effectiveness after reimmunization against GnRH was achieved, it would potentially provide a larger and more statistically powerful sample size of contracepted animals in which to detect behavioral changes related to this vaccine (if they occurred).

We completed an intensive behavioral study during an earlier phase of this larger GnRH study during 2009–2010 [37]. To make these analyses directly comparable to that prior study, we followed the same behavioral sampling design as that described previously [50]. Briefly, this included blocking observations into three daylight time periods (08:00–12:00 h, 12:01–16:00 h, and 16:01–20:00 h) with observations conducted during the primary breeding season, 1 March– 1 August 2014. Each observation session included collection of a 20-min instantaneous scan sample of time budgets at 1 min intervals for each adult band member ( $\geq 1$  year old), and all-occurrence data collection for social interactions [37].

Primary behavior categories included feeding, resting, locomotion, maintenance, and social behaviors [51]. Social behavior data included herding, reproduction, agonism, harem-tending, and harem-social behavior, and were collected at all occurrences throughout the observation sessions. Harem-social behavior was not collected through all-occurrence sampling in our previous study; however, it was collected during the scan sample in the previous study and was worthy of further consideration here. We defined this category as interactions between two individuals that did not meet the definition of the other all-occurrence behaviors (e.g. allogrooming and non-reproductive olfactory investigation).

We observed all horses from the nearest distance that did not elicit attention to the presence of the observer, typically 50–200 m. All observations were conducted using a 15–45  $\times$  600 mm spotting scope or 10  $\times$  42 mm binoculars when the distance between horses and observers was too far to allow unassisted detailed observation. We observed each band of horses weekly or bi-weekly in conjunction with other field assessments.

**Physiological.** Concurrently with foaling and behavior observations, we evaluated and compared potential adverse side-effects of treatment on injection-site reactions, body condition, success of existing pregnancy, and neonatal survival in treated and control mares. We made assessments of these potential side-effects monthly during the primary foaling season and opportunistically for the remainder of the year. We observed each mare for the presence or absence of visible lesions, swellings, or discharge at the injection site. In addition, we documented evidence of lameness (e.g. limping, gait alteration, reluctance to stand or bear weight on a limb), as well as behavioral depression, muscle tremors, or other systemic reactions that could be related to the vaccine treatment. We classified injection-site reactions according to the following criteria: 1) **abscess**—an open sore usually with fluid drainage or discharge, 2) **swelling**—a raised area of tissue of variable size and shape with no visible fluid drainage, 3) **lameness**—any abnormal range of movement or stiffness in the leg where the vaccine injection was delivered, 4) **none**—no observable reaction [52]. These categories were not mutually exclusive with respect to a single observation and both sides of the animal were observed, when possible. For these observations, we approached as near as possible to individual horses ( $\leq 50$  m) and assessed and photographed each injection-site reaction for later evaluation. At the same time, we visually evaluated body condition of each mare and scored condition as previously described [53]. We evaluated the success of the existing pregnancy by comparing foaling proportions between treated and control groups in 2010 and 2014. We measured neonatal survival as the proportion of foals surviving to 14 days of age and post-natal survival to 200 days.

## Statistical analysis

**Reproduction.** Yearly foaling data are reported as the proportion of mares observed with a foal in each group. We used asymptotic approximation to the binomial distribution to compute 95% confidence intervals for these proportions using package *binom* in program R [45, 47]. We used a risk ratio analysis ( $\alpha = 0.05$ ) to compare all observed annual proportions between treatment groups. We used the same method to evaluate the success of the existing



pregnancy between groups during the first foaling season post-vaccination (2010 and 2014). All comparisons between treatment groups were made within a single year and without multiple testing corrections.

**Behavior.** We used the same statistical approach for the analyses in 2014 as that used in 2010 [37]. We modeled the frequency of each behavior using mixed-effects linear regression, where individual female identity and sampling time (time of day) were included as random effects on the intercept term of each model. This accounted for variation that may have been present among individuals who were sampled repeatedly, though not always equally over time, and for temporal variation in behavior when samples were not equally collected across all times of the day. Time budget behaviors sampled at 1-min intervals were aggregated into proportion of time spent per behavior to calculate an independent measure of behavior per observation session. We used the *lme4* package of R version 3.1.2 (The R Foundation for Statistical Computing 2014) and SYSTAT 12.02.00 (SYSTAT Software, Inc. 2007) to calculate descriptive statistics and obtain mixed-effects model estimates using restricted maximum likelihood [54]. Separate models were fitted for each time budget behavior with the fixed effects of treatment group (treated or control), foal presence (dependent foal < 1 year of age present with the female, or no foal present with the female), female age, and band size. In the previous study, we considered band fidelity (number of times a female moved bands within a year), but data were too homogeneous to consider that factor in 2014: only 8 horses moved bands at all (4 treated/4 saline) and five of those moved collectively to a different stallion.

**Physiological.** We used descriptive statistics (arithmetic means with  $\pm$  95% CI) to compare, occurrence of lesions at the injection site and 1-tailed Fisher's exact test ( $\alpha = 0.05$ , 1df) to compare foal survival proportions of treated females to that of controls. We used normal binomial distributions to compute confidence limits for the differences between proportions using Jeffrey's interval for small sample sizes [55]. Effects on body condition scores were examined using generalized linear models in the *lmer* package in program R [56]. We employed random effects for year and individuals and then compared this nested model to full models which added the effect of either treatment or foaling using an ANOVA.

This research was approved by the Institutional Animal Care and Use Committees of the National Park Service (NPS) (Permit Numbers: MWR\_THRO\_Baker\_Horse\_2013.A3, MWR\_THRO\_Baker\_Horse\_2015.A3) and Colorado State University (IACUC Protocol No. 17-7651A). This study was conducted in accordance with good laboratory practices (GLP) and oversight from United States Department of Agriculture/National Wildlife Research Center (No.QA1647). All data collections were conducted after obtaining a scientific collection permit issued by Theodore Roosevelt National Park (THRO-2010-SCI-0010). All work, other than animal handling and vaccination at the two feral horse roundups, was observational. Every effort was made to prevent and minimize disruption of natural band dynamics and individual horse behavior and well-being during handling and treatment application.

## Results

The statistical process used to select experimental mares for this investigation resulted in two treatment groups that were relatively homogeneous in age, body condition, body mass, and pregnancy status [S1 Table]. Results of pregnancy assessment indicated that most mares were pregnant at the 2009 (0.86 (49/57), 95% CI = 0.74–0.93) and 2013 (0.90 (46/51), 95% CI = 0.79–0.96) roundups, thus providing sufficient opportunity to evaluate and compare the safety and potential side effects of vaccine treatment on pregnancy and neonatal survival.

Transrectal ultrasonography revealed that the fetuses of most pregnant females were approximately 120+ days old at the roundup and that most had descended over the pelvic rim

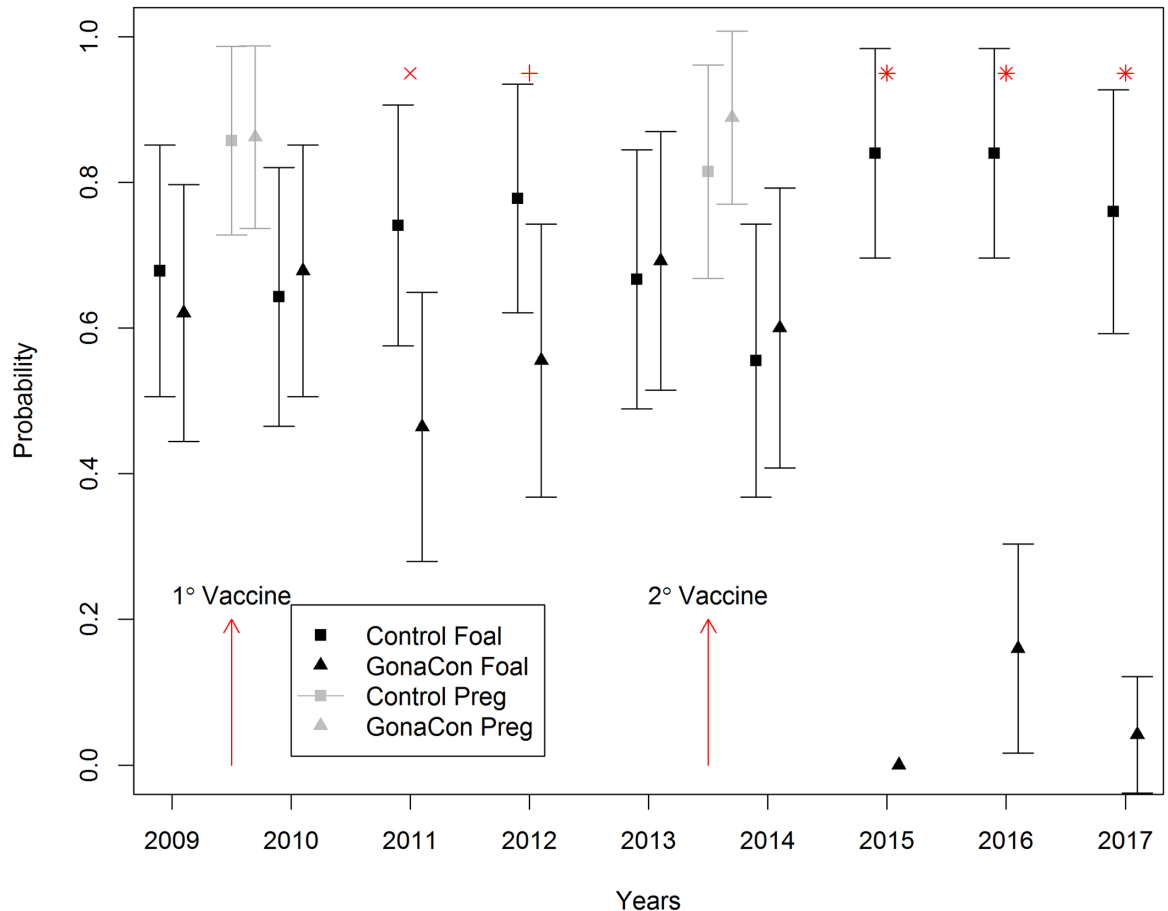
preventing a more accurate assessment of gestational age at treatment application [57]. To provide a more precise estimate, we used an estimated gestation period for horses of 342 days [58] and the approximate foaling date ( $\pm 5$  days) of each mare in 2010 and 2014 and then back-calculated to the date of treatment application at the 2009 (18–23 October) and 2013 (23–25 September) roundups. Using these calculations, we estimated mean gestational age at vaccine inoculation in 2009 to be 162 days (95% CI = 150–175) for treated mares and 154 days (95% CI = 138–170 days) for control mares. For 2013, we used the same calculation and projected that, on average, females were reimmunized against GnRH at approximately 129 days (95% CI = 105–151 days) of gestation and saline-treated control mares at 132 days (95% CI = 119–144 days).

Following the 2009 and 2013 roundups and release, experimental mares distributed themselves among 16–19 individual bands. At least one treated or control mare was present in all bands during 2010–2017. Likewise, the composition of adult mares in each band, as well as the band stallions, remained relatively stable during this period. By the end of the 2017 foaling season, 14% (4/29) of treated mares and 11% (3/28) of control mares had died of various causes (e.g., malnutrition, broken appendage, dystocia, unknown causes). Except for these mares and one vaccinated mare that was not re-captured at the 2013 gather, all others were observed for foaling and other field measurements for all eight years of this investigation.

We met our sampling objective by observing more than 95% of all mares weekly (and sometimes more often) from 1 March to 1 August each year of the study. It is possible that some foals were born and died without being detected but given the intensity of the sampling observations, we feel that this was highly unlikely. Observations during the remainder of the year and following winter were less intense and more opportunistic depending upon available personnel, weather, and road conditions. During this time, mortality of foals was more likely to have gone undetected.

## Vaccine effectiveness

**Primary vaccination (2009–2013).** Mean foaling proportions of treated (0.62 (18/29) 95% CI = 0.44–0.79) and control (0.68 (19/28) 95% CI = 0.50–0.85) mares during the 2009 pre-treatment foaling season were not different ( $P = 0.65$ ) indicating that prior to contraception, treatment groups exhibited equal fertility [S1 Table]. Further evidence was provided by individual mares at the 2009 gather and primary vaccine inoculation. The proportion of treated (0.86 (25/29), 95% CI = 0.71–0.95) and control (0.85 (24/28), 95% CI = 0.70–0.95) mares determined to be pregnant, via transrectal ultrasonography, were not different ( $P = 0.63$ ) [S1 Table, Fig 1]. This provided an opportunity to compare the effects of GonaCon-Equine vaccination on the existing pregnancy of treated mares and neonatal health and survival to that of untreated control mares. Foaling proportions of treated (0.68 (19/28) 95% CI = 0.50–0.85) and control (0.64 (18/28), 95% CI = 0.46–0.82) mares during 2010 were not different ( $P = 0.78$ ) (Fig 1). Births occurred from early March to early September with 97% (35/36) observed during the first four months of the foaling season (1 March to 1 June). Average foaling dates in 2010 for treated and control mares were 5 May (95% CI = 22 April–18 May) and 10 May (95% CI = 25 April–25 May), respectively. No foal was detected for 12 mares (6 treated: 6 control) that were determined to be pregnant at the 2009 gather. None of these mares showed evidence of pregnancy during the intensive foaling period or for the remainder of the year. We surmised that most of these foals were either aborted or died as neonates between the periods from 20 October 2009 (gather) to 1 March 2010 (beginning of foaling observations). Regardless of timing or cause of death, the proportion of mares that foaled in



**Fig 1. Comparative probability of foaling and pregnancy for treatment and control groups of free-ranging feral horses (*Equus caballus*) mares selected for this experiment.** Mares were treated with a primary vaccination of GonaCon-Equine in October 2009 and then reimmunized with the same vaccine in September 2013 at scheduled gathers at Theodore Roosevelt National Park, North Dakota, USA. GonaCon vaccinations occurred at the time points represented by the red arrows. Symbols correspond to observed p-values for relative risk comparisons between treatment groups within years (p-value between 0.05 and 0.1 = +, for < 0.05 = x, and for <  $1 \times 10^{-05}$  = \*).

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2010 underestimated the proportion of mares that were determined to be pregnant at the 2009 gather by 24% for treated mares and 21% for control mares.

Estimated age of all foals at first observation was 2.4 days (95% CI = 1.7–3.1 days). Most neonates (97%), from both experimental groups, were classified as vigorous and in good to excellent condition when first observed. Neonatal survival rate from parturition to 14 days of age was estimated to be 0.95 (18/19, 95% CI = 0.75–0.99) for foals born to GonaCon-treated females and 0.88 (16/18, 95% CI = 0.64–0.98) for foals born to control mares ( $P = 0.54$ ). After 14 days of age, post-neonatal survival rates (14–200 da) averaged 0.97 (30/31, 95% CI = 0.84–0.99) and were similar for both experimental groups ( $P = 0.57$ ). These results support our prediction ( $H_3$ ) that inoculation with GonaCon-Equine vaccine, during approximately the second trimester of pregnancy, does not affect the existing pregnancy of treated females or neonatal health and survival.

The proportion of treated mares that foaled (13/28) following a single vaccination was lower than that for control mares (19/26) for the second (2011) ( $P = 0.04$ ) and third (15/27 vs 21/27) (2012) ( $P = 0.08$ ) post-treatment foaling seasons but was similar (18/26 vs (18/27)

( $P = 0.67$ ) to control mares for the fourth (2013) season, demonstrating reversibility of the primary vaccine treatment (Fig 1). Even though we observed a significant reduction in foaling proportions between treated and control mares during 2011 and a declining effect in 2012, therapeutic effectiveness and relative risk reduction estimates were low to modest and estimated to be 0.37 (95% CI = 0.01–0.60) and 0.28 (95% CI = -0.06–0.51), respectively (Table 1). These findings lend support to our hypotheses ( $H_1$ ) that a single vaccination with GonaCon-Equine is reversible and suppresses fertility for multiple years post-treatment in a portion of treated animals but with diminished effectiveness over time.

**Secondary vaccination (2013–2017).** At the scheduled gather in October 2013, we extended our evaluation of GonaCon-Equine by assessing the effects of revaccination on fertility and safety in these same experimental mares treated four years after the primary vaccination. Evidence of similar fertility for individual mares was demonstrated at the 2013 gather, where pregnancy proportions of treated (0.92 (23/25), 95% CI = 0.75–0.98) and control (0.88 (23/26), 95% CI = 0.71–0.96) mares were similar ( $P = 0.86$ ) [S1 Table]. Except for one treated and one control mare, all others had conceived and given birth to at least one foal during 2009–2013. For the 2013 foaling season, foaling proportions of treated (0.69 (18/26), 95% CI = 0.51–0.87) and control (0.66 (18/27) 95% CI = 0.49–0.84) mares were not different ( $P = 0.84$ ) providing additional evidence that treatments groups were of equal fertility prior to reimmunization (Fig 1).

Like 2010, mean foaling proportions during the first post-treatment foaling season (2014) were not different ( $P = 0.74$ ) between treated (0.60 (15/25), 95% CI = 0.41–0.79) and control (0.56 (15/27), 95% CI = 0.37–0.74) mares (Fig 1) supporting similar observations in 2010 that revaccination could be applied to pregnant mares, during mid-gestation, without risk to the existing pregnancy. Foaling date distribution was comparable to that observed in 2010 following the primary vaccination. Average foaling date for treated mares was estimated to be 27 April (95% CI = 5 April– 20 May) and 19 April (95% CI = 6 April– 2 May) for controls. No foal was observed for 15 mares (8 treated: 7 control) that were determined to be pregnant at the 2013 gather. Like 2010 estimates, foaling proportions underestimated pregnancy proportions determined at the 2013 gather for both treated and control mares by approximately 30% and 34%, respectively (Fig 1). These data, together with similar observations in 2010, support the inference that foaling proportions are not an accurate proxy for pregnancy proportions but provide a limited but practicable field measurement for determining contraceptive

**Table 1. Comparative relative risk reduction (RRR), 95% confidence intervals, and p-values associated with differences in foaling proportions between GonaCon-treated and control mares during 2009–2017.**

Year	Relative Risk Reduction	95% Confidence Interval		p-value
	(RRR)	Lower	Upper	
2009	0.0852	0.3757	-0.3402	0.6500
2010	-0.0555	0.2750	-0.5301	0.7797
<b>2011</b>	<b>0.3732</b>	<b>0.6028</b>	<b>0.0109</b>	<b>0.0381*</b>
2012	0.2857	0.5178	-0.0581	0.0861
2013	-0.0384	0.2826	-0.5032	0.8430
2014	-0.08	0.3216	-0.7194	0.7482
<b>2015</b>	<b>1</b>	<b>1</b>	<b>NA</b>	<b>2.57E-09*</b>
2016	0.8095	0.9236	0.5247	1.94E-06*
2017	0.9451	0.9920	0.6217	4.15E-07*

\*Significant p-values (<0.05).

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effectiveness. Average foal age at first observation across both treatment and control groups was 2.6 (95% CI = 1.5–3.3) days. Nearly all foals born to revaccinated and control mares were classified as vigorous and found to be in good to excellent condition when first observed.

Neonatal survival rate to 14 days of age for foals born to revaccinated mares was 0.87 (13/15), 95% CI = 0.62–0.96 and 0.93 (14/15), 95% CI = 0.70–0.98) for foals born to control mares ( $P = 0.49$ ). After 14 days of age, post-neonatal survival rates were 0.80 (12/15), 95% CI = 0.55–0.92) for revaccinated mares and 0.73 (11/15), 95% CI = 0.48–0.89) for control mares ( $P = 0.55$ ). These results reflect similar findings following a primary vaccination with GonaCon-Equine and reinforces the deduction ( $H_3$ ) that reimmunization is safe for treatment of pregnant females and does not affect neonatal or post-neonatal health or survival when applied at approximately mid-gestation.

Unlike results from the single vaccination trial, we observed, not only highly significant reduction in foaling proportions between treated and control mares following reimmunization but also a remarkably effective contraceptive response. Except for the first foaling season following treatment application, (2014) in which the vaccine was not expected to have an effect ( $P = 0.75$ ), foaling proportions in reimmunized mares were lower ( $P < 0.001$ ) than that for control mares for all subsequent years (2015–2017) (Fig 1). This was particularly evident for the second post-treatment foaling season (2015) when none 0.00 (0/25), 95% CI = 0.0) of the reimmunized mares produced a foal while the proportion of control mares foaling was estimated to be 0.84 (21/25, 95% CI = 0.69–0.98). During the third post-treatment foaling season (2016), four treated mares produced a foal resulting in a foaling proportion of 0.16 (4/25), 95% CI = 0.01–0.30) while the proportion of control mares foaling was identical to that observed in 2015 (Fig 1). These foals were determined to be vigorous and in good to excellent condition at birth, however, two of these foals, born in September, were not observed the following spring and were categorized as post-natal mortalities and presumed to have died during winter (2016/2017).

In 2017, no additional treated mares produced a foal or showed evidence of pregnancy. However, one of the treated mares that had foaled in 2016 died of apparent natural causes (age-related malnutrition) during 2017 and two other revaccinated mares that had foaled in 2016 failed to produce a foal that year resulting in a foaling proportion of 0.041 (1/24), 95% CI = 0.03–0.12) (Fig 1). The foaling proportion for mares in the control group (2017) was 0.84 (21/25, 95% CI = 0.69–0.98) and higher ( $P < 0.001$ ) than that for GonaCon-treated mares (Fig 1). It should be noted that the apparent decrease in foaling proportions in GonaCon-treated mares from 2016–2017 and resulting increase in vaccine effectiveness (Table 1) is likely due to the inherent error associated with the small sample size ( $n = 4$ ) of mares in this treatment group that regained fertility. Overall, there was both a substantial decrease in foaling proportions (Fig 1) and an exceedingly high level of effectiveness (Table 1) for treated mares compared to controls for 3 years post-revaccination (2015–2017) ( $P < 0.001$ ). Thus, fertility measurements during 2015–2017 support our prediction ( $H_2$ ) that revaccination with GonaCon-Equine would be more effective in suppressing foaling proportions in treated females compared to controls than a single immunization (Fig 1, Table 1).

## Side effects

**Behavioral.** We collected behavioral data on 73 feral horses (22 males, 25 treated females, 26 saline females) for 218.3 h in 2014. The median age of observed stallions was 12 years (range = 9–19 years), median age of observed control females was 8 years (range = 7–20), median age of observed treated females was 9 years (range = 7–22), and median band size was 8 horses (range = 2–14). There were no differences detected between treatment groups in any

time budget behavior category (Table 2). As band size increased, feeding decreased 1.24% (95% CI = 0.48–2.00) per additional horse in the band. Likewise, locomotion increased 0.20% (95% CI = 0.07–0.33) and maintenance decreased 0.10% (95% CI = 0.01–0.19) per additional horse in the band.

Foal presence influenced locomotion, with barren females moving 1.24% (95% CI = 0.40–2.08) more than females with dependents. Foal presence also influenced the social behavior component of time budgets, with barren females interacting with others 2.74% (95% CI = 0.59–4.90) more than females with dependents.

Variance among individuals had little influence on any of the behaviors modeled (Table 2). Variance was also minimal between time periods of observation; however, there were some significant differences in amount of activity by time of day. An estimated 6.88% (95% CI = -0.73–14.5) more feeding occurred in the 1601–2000 h time-period than did earlier in the day, and this was reciprocated by an estimated 3.33% (95% CI = 1.09–7.79) less resting, 0.34% (95% CI = 0.15–0.82) less maintenance, and 1.30% (95% CI = 0.51–3.11) less social behavior during the same period.

There were no differences detected between treatment groups in herding, reproduction, or agonism, but treatment group did influence harem-social behavior. Observed instances of harem-tending behavior provided too few data to model. Because these social behaviors were not as dependent on other broad categories as is the case with compositional time budgets [51], we re-estimated the social behavior models with only treatment and supported effects to allow for clearer interpretation of the results.

Stallions initiated harem-social behavior 13.9% (95% CI = 3.25–24.68) less toward control females than toward treated females. Though all harem-social records were analyzed as a group, it should be noted that 55.8% of the 308 harem-social events were sub-categorized as allogrooming. While the significant difference between treatment groups was detected, the variance among individuals for this behavior was near zero (Table 2).

**Physiological.** No study mares exhibited antibody titers to any of the infectious diseases that were surveyed for (i.e., equine herpesvirus-1, equine infectious anemia, equine viral arteritis and contagious equine metritis) thus eliminating this factor as a potential cause of infertility in GonaCon-treated females.

No control mares, treated with saline, showed any evidence of injection site reactions. Swelling and discharge were never observed in this group. Likewise, these mares showed no evidence of lameness or gait abnormalities in either hind limb. Consistent with our hypothesis ( $H_3$ ), approximately 72% of treated mares (21/29) displayed a visible reaction at the site of injection after a single vaccination with GonaCon-Equine (S1 Photo). A single mare developed a draining abscess after the initial vaccination. These lesions were persistent over multiple years. At the time of the 2013 roundup and revaccination, 81% (21/26) of vaccinated mares continued to have palpable swelling at the original site of vaccine injection.

Like initial vaccination reactions, during the first-year post-revaccination, approximately 50% (13/26) of mares continued to show swelling on the left hip at the site of the 2009 injection and 50% developed a reaction on the right hip at the site of revaccination in 2013. Two of these new reactions were draining abscesses. Yet again, injection site reactions were persistent with approximately half of the mares with swellings at one or both injection sites, 3 years after revaccination. None of the GonaCon-treated mares displayed any evidence of lameness, altered gait or abnormal range of movement throughout the 8 years they were observed.

While body condition varied between individuals and study years, it did not vary between treatment groups ( $P = 0.14$ ) over the course of the study. Likewise, there was no effect of presence of a foal on body condition ( $P = 0.16$ ). Average body condition ranged from 3.7–4.9

**Table 2. Treatment and supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) time budget behaviors (e.g. feeding, resting, locomotion, maintenance, social) and all-occurrence social behaviors (e.g. herding, reproduction, agonism, harem- social) at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day (j) and individual horse identity (k) are shown as  $\sigma_j^2$  and  $\sigma_k^2$ .**

Behavior	Effect	t	P	Difference	95% confidence limit		$\sigma_j^2$	$\sigma_k^2$
					Lower	Upper		
Feeding	Treatment	-0.125	0.900				0.004	0.003
	Band Size	-3.193	0.001	-0.012	-0.020	-0.005		
Resting	Treatment	0.590	0.555				0.001	0.001
Locomotion	Treatment	-0.143	0.886				<0.001	<0.001
	Band Size	3.047	0.002	0.002	0.001	0.003		
	Foal Presence	2.900	0.004	0.012	0.004	0.021		
Maintenance	Treatment	-1.193	0.233				<0.001	<0.001
	Band Size	-2.238	0.025	-0.001	-0.002	-0.001		
Social	Treatment	-0.037	0.970				<0.001	0.001
	Foal Presence	2.499	0.013	0.027	0.006	0.049		
Herding	Treatment	-0.909	0.368				0.009	<0.001
Reproduction	Treatment	1.555	0.159				<0.001	<0.001
Agonism	Treatment	0.669	0.528				<0.001	0.048
Harem-social	Treatment	2.620	0.012	0.140	0.033	0.247	0.007	<0.001

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(moderately thin to moderate body condition) for all study animals over the 8 years that mares were observed. Individual body condition scores ranged from 1–7.

## Discussion

### Reproduction

This study demonstrated that a single vaccination against GnRH, using GonaCon-Equine, administered during mid-gestation, was safe, initiated short duration (2 yrs.) infertility in some mares, and was reversible, but was minimally effective in reducing fertility of treated females compared to controls. For two foaling seasons following vaccine treatment, we observed statistically significant reductions (28–38%) in foaling proportions of treated versus control mares but no effect by the third-year post-treatment, thus confirming the reversibility of the vaccine.

These results parallel similar findings from other experimental evaluations of GonaCon-Equine reported for captive and free-ranging mares. In a comparable study in Nevada with feral horses in a natural environment, GonaCon-Equine reduced foaling proportions by an average of 33% over a 3-year period but, like our study, contraception was only modestly effective over this period [23]. In contrast, contraceptive effectiveness of captive mares treated with GonaCon was greater and longer lasting ( $\geq 4$  yrs) than either of these studies [22]. The disparity between captive and free-ranging animals in contraceptive response to GonaCon vaccine is not limited to feral horses but has also been observed between captive and free-ranging white-tailed deer [14, 18, 20] and elk [15, 16, 19]. Although these investigations did not suggest a definitive causation for these differences, they all pointed to suppressed and less persistent GnRH antibody concentrations in free-ranging ungulates compared to their captive counterparts suggesting a relatively compromised or weakened immune response to the vaccine that resulted in reduced contraceptive effectiveness.

It is widely acknowledged that differences in vaccine effectiveness can be attributed to increased environmental stressors (i.e., nutritional status, injuries, parasite load, pathogen exposure, and social dynamics) that can inhibit a more vigorous immune response in free-

ranging animals in a natural environment [59, 60]. It follows that while efficacy trials with captive animals can provide an important first approximation of vaccine safety and performance under controlled conditions; they may offer only limited inference to free-ranging animals that are not buffered against natural stressors that may decrease immune response and vaccine effectiveness. Regardless of the factor(s) contributing to the limited effectiveness of GonaCon in free-ranging animals, it appears that the immune response from a single vaccination does not consistently provide multiple years of infertility in all or even a high proportion of these animals.

In comparison to a single inoculation with GonaCon-Equine, the effect of reimmunization on foaling proportions was highly significant which allowed clear differentiation between treated and control mares for multiple breeding seasons. Compared to a single vaccination, reimmunization of mares in this study resulted in a much higher (58%) average vaccine effectiveness (range = 0.80–0.94) than the single vaccination for a 3-year period (2015–2017). Likewise, this level of effectiveness following reimmunization was on average higher than that previously reported for free-ranging mares treated with a single application with GonaCon-Equine [23] and 32% above what was reported for captive mares treated with the same vaccine formulation [22]. These results support the conclusion that a booster immunization with GonaCon-Equine can provide a highly effective, multi-year suppression of fertility in free-ranging horses and these results may be consistent in other animal species, as well.

It is fundamental knowledge that a secondary response to a vaccine generally results in a more rapid production of antibodies that are produced in greater amounts and over a longer time compared to the primary vaccination [25]. Repeat immunizations using a variety of GnRH vaccines in domestic horses have been shown to improve contraceptive efficacy. However, unlike commercially available short duration vaccines (< 1 yr.) developed for domestic horses [29, 61], GonaCon-Equine is formulated by combining a non-biodegradable oil in water-based emulsion and an optimum concentration of immunostimulatory killed mycobacteria to form a depot usually deep in muscle tissue. This depot injection is thought to allow for a slow release and prolonged stimulation so that the formulation can act for much longer periods of time (years) than is possible with standard injections (months). This effect is thought to be responsible for the extended antibody response of 3–4 years in vaccinated deer [14, 18, 20, 62], elk [15, 16], and horses [22].

While this response was not unexpected, the magnitude and duration of effectiveness of GonaCon-Equine following revaccination, even 4 years after the initial vaccination, is salient and relevant to the management of fertility in free-ranging horses. First, it demonstrates that a booster vaccination can stimulate a highly effective immune response that can result in multiple years ( $\geq 3$  yrs.) of contraception. Second, it provides an initial reference point for defining the optimum revaccination schedule required for long-term reproductive management of female horses in a natural environment. And finally, it supports the consideration that while a single application may be preferred from a practical management perspective, GonaCon-Equine is more effective, in free-ranging horses, if repeat vaccinations are delivered on a periodic basis. While initial results are encouraging, additional research is needed to complete the objectives of this study including: 1) to define the duration of effective contraception post-revaccination, 2) to determine if long-term or permanent infertility is a possible outcome, and 3) to assess if return to fertility (if it occurs) results in altered birth phenology of treated mares. We will investigate these questions over the next three years of this study. Additionally, there may be a more optimal revaccination schedule which allows for altered duration of effectiveness or is more conducive to management schedules.



## Side effects

After revaccinated in October 2013, time budget and social behaviors of mares in spring/summer of 2014 were comparable to those observed during the same period in 2010, following the initial treatment in October 2009. We found no evidence of differences in frequency or intensity of social behaviors including estrous behavior associated with treatment. Both treatment and control groups displayed few estrus behaviors in either 2010 [37] or during 2014. Behaviors associated with estrus were observed only 17 times in treated and 57 times in control mares out of 1148 observed social behavior events. This supports our earlier findings that pregnant mares rarely show overt estrous-related behaviors and similarly GonaCon-Equine treated mares only occasionally display these behaviors, although each for different reasons. Once a mare is pregnant, progesterone likely subverts much of the estrous type behavior that would generally be displayed with high estrogen levels, and only occasionally do domestic horses display and stand for mounting when pregnant [63]. Relatively small amounts of estrogen are secreted as follicles develop and then regress. In the absence of progesterone, relatively small amounts of estrogen are likely sufficient to induce erratic estrous behavior as was observed in these mares. However, the small amounts of estrogen were likely insufficient to induce an LH surge and subsequent ovulation.

Regardless of the underlying endocrinology associated with these behaviors, vaccinated and control mares both displayed social interactions that maintained herd structure; herding, tending, and defending behaviors from the stallion; and social hierarchies. The only meaningful factor that influenced the amount of time spent in social behaviors (e.g. allo-grooming, herding and tending) was the presence or absence of a foal. Mares with foals spent more time alone with the foal than those without off-spring, which is to be expected given their social and nutrient requirements during the neonatal and post-natal periods [50]. It is possible that long-term absence of foals could influence social behavior on a longitudinal scale, but additional studies are needed to investigate such phenomena on an appropriate time scale.

Other techniques for reducing the fertility of free-ranging species, such as vaccination with the native porcine zona pellucida vaccine (PZP) and tubal ligation, maintain the competency of the endocrine aspects of fertility. This can lead to unintended consequences with repeated estrous cycling in polyestrous species. In fact, in a population of white-tailed deer, where most reproductive females had received tubal ligations, fawning was negligible; however, there was more than a 700% increase in the number mature males attracted to the area occupied by a high number of estrous cycling females [64]. Similarly, PZP vaccination has extended the length and intensity of breeding seasons in horses [49, 65–68], deer [69, 70], and elk [71]. GonaCon-Equine may avoid these inadvertent consequences by functionally inducing mimicry of pregnancy in females which continues to be an important part of the social structure of the group but does not invite intense adverse breeding behaviors.

Researchers have generally hypothesized that by alleviating the energetic demands of gestation and lactation, contracepted females will attain improved body condition over pregnant females that require additional food resources to produce and rear an offspring. However, for free-ranging large ungulates, empirical evidence supporting [72] or refuting [73–75] this prediction is limited and equivocal. In this investigation, contracepted mares that experienced no gestation and lactation did not exhibit improved body condition over mares that successfully reproduced. Individual mares in each experimental group, attained an average BCS of 5.0 (moderate) or better, which has been reported to be the minimally optimal level of stored fat necessary to achieve maximum reproductive efficiency during pregnancy and lactation [53, 76]. These levels of body condition were reflected in the high proportion of pregnant mares (0.85–0.92) observed in each treatment group at the management roundups in 2009 and 2013.

We acknowledge that our sampling intensity and/or sensitivity of our ocular index to body condition may not have enabled us to detect fine-scale differences between experimental groups. However, we conducted these evaluations during time periods when differences in body condition between pregnant and non-pregnant (GonaCon-treated) females should have been the greatest. Namely, during early spring (March) when fats deposits are depleted over winter and during April–August when the energetic demands of late gestation and lactation are increasing.

The body condition of an animal is dependent on a balance between energy intake and expenditure. When intake is not sufficient to meet energy requirements for various activities (i.e. maintenance, growth, activity, gestation, lactation, etc.), fat reserves and eventually lean body tissue will be lost. The fact that pregnant and lactating mares in this study were in similar body condition to that of contracepted ones suggest that food is unlikely a limiting factor for free-ranging horses at THRO. This is primarily due to the conservative management of multiple species of ungulates and their food resources [77–79]. The consequence of this approach is that only under the most extreme climatic conditions, such as prolonged drought, will forage be limiting to herbivores at THRO, regardless of reproductive status.

The only detectable adverse side effect of vaccination was intramuscular swelling at the vaccination site. Mares treated with GonaCon-Equine consistently showed evidence of inflammatory reactions at the injection site. While we never observed lameness associated with this reaction, several mares revealed draining abscesses within one-year post-vaccination. This is consistent with results for other wild ungulates treated with the same or similar GonaCon vaccines [13, 15, 34]. Given the designed highly inflammatory nature of both the adjuvant, which contains killed mycobacteria and non-biodegradable oil, as well as, the foreign protein carrier molecule, these types of reactions are predictable. In fact, they are likely necessary for optimum vaccine efficacy [80]. It is impossible to assess the total impact of these lesions on animal welfare; however, in this investigation, these did not have a measurable effect on body condition, locomotion, or social behaviors. Therefore, until additional research suggests otherwise, we conclude that the presence of injection site lesions following GonCon vaccination do not pose a serious contraindication associated with the application of this vaccine, and there appear to be minimal long-term effects on individual animal welfare.

## Conclusions

Controlling abundance of wildlife species that are simultaneously protected, abundant, competitive for resources, and in conflict with some stakeholders is a formidable challenge for resource managers. We demonstrated that the GnRH vaccine, GonaCon-Equine, could be an effective immunocontraceptive for free-ranging feral horses, particularly when the primary vaccination is followed by reimmunization four years later. This vaccine was shown to be safe for pregnant females and neonates and did not result in deleterious behavioral side effects during the foaling/breeding season. The only adverse reactions to vaccination were non-debilitating inflammatory responses at injection sites. One noteworthy implication has emerged regarding long-term management of free-ranging horse populations using GonaCon-Equine vaccine: effective management and development of population models will need to incorporate repeat immunizations of this vaccine to optimize management strategies aimed at stabilizing the growth rate of feral horse populations. Our research suggests that practical application of this vaccine in feral horses will require an initial inoculation that may provide only modest suppression of fertility followed by reimmunization over time that together could result in greater reduction in population growth rates. Future research will begin to define the most effective revaccination schedule with GonaCon-Equine for suppressing reproductive rates in

free-ranging horses, the duration of effectiveness, and the return to fertility following treatment. Moreover, applying GonaCon-Equine to control the growth of feral horse populations will require that resource managers choose specific tactics for treating animals. Choices must be made on the number and age to treat and the frequency of treatment needed to maintain the desired population age structure and genetic diversity. Decisions on the most beneficial tactics will depend on overarching management goals and long-term objectives for the population.

## Supporting information

**S1 Table. Comparative metrics and pregnancy proportions, by age class, for treatment and control groups of free-ranging mares selected for this experiment.**

(PDF)

**S1 Photo. An Intramuscular swelling (raised area of tissue) observed in a Theodore Roosevelt National Park horse, 9 months after hand-injection with GonaCon-Equine vaccine.**

(TIF)

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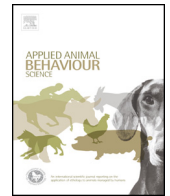
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## Behavior of feral horses in response to culling and GnRH immunocontraception



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### ABSTRACT

Wildlife management actions can alter fundamental behaviors of individuals and groups, which may directly impact their life history parameters in unforeseen ways. This is especially true for highly social animals because changes in one individual's behavior can cascade throughout its social network. When resources to support populations of social animals are limited and populations become locally overabundant, managers are faced with the daunting challenge of decreasing population size without disrupting core behavioral processes. Increasingly, managers are turning to fertility control technologies to supplement culling in efforts to suppress population growth, but little is quantitatively known about how either of these management tools affects behavior. Gonadotropin releasing hormone (GnRH) is a small neuropeptide that performs an obligatory role in mammalian reproduction and has been formulated into the immunocontraceptive GonaCon-B™. We investigated the influences of this vaccine on behavior of feral horses (*Equus caballus*) at Theodore Roosevelt National Park, North Dakota, USA, for a year preceding and a year following nonlethal culling and GnRH-vaccine treatment. We observed horses during the breeding season and found only minimal differences in time budget behaviors of free-ranging female feral horses treated with GnRH and those treated with saline. The differences observed were consistent with the metabolic demands of pregnancy and lactation. We observed similar social behaviors between treatment groups, reflecting limited reproductive behavior among control females due to high rates of pregnancy and suppressed reproductive behavior among treated females due to GnRH-inhibited ovarian activity. In the treatment year, band stallion age was the only supported factor influencing herding behavior ( $P < 0.001$ ), harem-tending behavior ( $P < 0.001$ ), and agonistic behavior ( $P = 0.02$ ). There was no difference between the mean body condition of control females (4.9 (95% CI = 4.7–5.1)) and treated females (4.8 (95% CI = 4.7–4.9)). Band fidelity among all females increased 25.7% in the year following vaccination and culling, despite the social perturbation associated with removal of conspecifics. Herding behavior by stallions decreased 50.7% following treatment and culling ( $P < 0.001$ ), while harem-tending behavior increased 195.0% ( $P < 0.001$ ). The amount of available forage influenced harem-tending, reproductive, and agonistic behavior in the year following culling and treatment ( $P < 0.04$ ). These changes reflected the expected nexus

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between a species with polygynous social structure and strong group fidelity and the large instantaneous change in population density and demography coincident with culling. Behavioral responses to such perturbation may be synergistic in reducing grazing pressure by decreasing energetically expensive competitive behaviors, but further investigation is needed to explicitly test this hypothesis.

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## 1. Introduction

Gregarious relationships can impart individual benefits such as decreased depredation, increased foraging efficiency, and increased fecundity (Pusey and Packer, 1997). When resources to support populations of social animals are limited and populations become locally overabundant, managers are faced with the daunting challenge of decreasing population size without disrupting core behavioral processes. For example, the limited contiguous habitat and finite resources found on public wildlands cannot sustain overabundant populations of large ungulates, and managers continuously seek innovative and publically-acceptable tools for managing these species (Powers et al., 2011). Historically, such populations have been reduced by culling, using either lethal methods such as hunting, or non-lethal methods such as capture and translocation. Limiting fecundity through the use of immunocontraceptives is becoming a more commonly considered tool for controlling wildlife abundance. However, these tools can be accompanied by physiological changes that may alter behavior and ultimately influence population dynamics in unforeseen ways (Ransom et al., 2014). The paucity of quantitative data on natural behavior of wild free-roaming fauna, especially pertaining to those treated with fertility control agents, impairs our ability to understand species' influences and roles in ecosystems and thus our ability to effectively manage populations.

Elk (*Cervus canadensis*) and feral horses (*Equus caballus*) are increasingly targeted for fertility control management in the United States and both species exhibit complex social behavior and polygynous mating systems. Whereas elk congregate seasonally into mating groups (Geist, 1982), feral horses form bands that persist year-round with distinct associations of individuals sometimes lasting a decade or more (Klingel, 1982). These bands are largely maintained by reproductive, herding, agonistic, and defensive behaviors initiated by the polygynous male in the band, as well as hierarchical relationships among females (Ransom and Cade, 2009). Culling can result in discrete changes to population density and demography, which could perturb social organization. Likewise, effective fertility control agents lead to discrete changes in presence of offspring in the population, and thus may influence social behavior.

Approximately 33,000 feral horses roam 13 million ha of public lands in the western United States and managers have historically relied on nonlethal culling through a process of capture, removal, and public adoption to regulate animal abundance (Garrott and Oli, 2013). This process is unsustainable because demand in the U.S. for feral horses as domestic stock is less than half the number of animals

removed, which has left as many horses in federally-subsidized pastures as persist in the wild. Several fertility control techniques have been investigated for use in feral horses, but most of these efforts resulted in tools that were publicly unacceptable, expensive to use, or impractical on a large scale. Among the most promising techniques are immunocontraceptive vaccines such as porcine zona pellicida (PZP) and gonadotropin-releasing hormone (GnRH). These agents are relatively easy to apply, are reasonably efficacious, and are not expected to be accompanied by serious contraindications (Kirkpatrick et al., 2011).

Gonadotropin releasing hormone is a small neuropeptide that performs an obligatory role in mammalian reproduction. When combined with a potent adjuvant, the GnRH vaccine stimulates a persistent immune response resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant (Miller et al., 2008). The most compelling hypothesis of vaccine effectiveness suggests that antibodies to GnRH likely induce transient infertility by binding to endogenous GnRH, thus preventing attachment to receptors on gonadotropes and suppression of pulsatile luteinizing hormone (LH) secretion (Molenaar et al., 1993). Gonadotropin releasing hormone antibody titers are correlated with suppression of the reproductive system and infertility in a variety of species (Fagerstone et al., 2010). As anti-GnRH antibodies decline over time, concentrations of available endogenous GnRH increase and treated animals usually regain fertility (Powers et al., 2011).

This immunocontraceptive vaccine, known by the trade name as GonaCon-B™, has been shown to provide multiple years of infertility in several wild ungulate species including horses (Killian et al., 2008; Gray et al., 2010), bison (*Bison bison*) (Miller et al., 2004), elk (Powers et al., 2011), and white-tailed deer (*Odocoileus virginianus*) (Gionfriddo et al., 2009). Given the physiological mechanism of action, GnRH immunocontraception has the potential to reduce fertility and suppress the reproductive behaviors typically associated with copulation; however, in GnRH-vaccinated elk, such behaviors were not entirely diminished likely due to incomplete suppression of the hypothalamic pituitary gonadal (HPG) axis (Powers et al., 2011). Similar to elk, feral horses are polyestrous and fertility control agents that do not suppress these social behaviors can lead to females exhibiting frequent reproductive behaviors that can cascade into altered behaviors from associated band members and nearby males (Nuñez et al., 2009; Ransom et al., 2010).

Rigorous quantitative investigation into the potential effects of GnRH treatment on feral horse behavior is missing from the assessment of this immunocontraceptive as a potential management tool. Such data are

**Table 1**

Population metrics for feral horses (*Equus caballus*) at Theodore Roosevelt National Park, North Dakota, USA, 2009 and 2010. Normalized Difference Vegetation Index (NDVI) is shown for only the behavior observation period in each year.

	2009	2010
Number of males	64	26
Number of females	74	59
Number of foals	27	31
Mean male age $\pm$ SE (range)	5.0 $\pm$ 0.45 (1–15)	7.7 $\pm$ 0.68 (1–16)
Mean female age $\pm$ SE (range)	5.3 $\pm$ 0.50 (1–20)	6.4 $\pm$ 0.45 (1–18)
Number of bands	19	16
Mean band size $\pm$ SE (range)	8.3 $\pm$ 0.07 (3–14)	8.7 $\pm$ 0.07 (3–19)
Total population size	165	116
Mean NDVI (95% CI)	0.44 (0.324–0.548)	0.43 (0.346–0.504)
Density (number of horses/ha)	0.009	0.006

also absent in regard to culling, which is arguably the most common feral horse management practice worldwide. We investigated the influences of GnRH treatment on behavior of feral horses at Theodore Roosevelt National Park, North Dakota, for a year proceeding and a year following GnRH vaccination and culling. Our objectives were to quantify effects of these two management tools on time budgets of individuals and social interactions between individuals. Based on the assumed mechanism of action of GnRH vaccine, we posited that inoculation would suppress reproductive behaviors in treated females compared to controls and that the sudden decrease in density and social perturbation caused by population reduction would measurably influence social behaviors of all horses.

## 2. Materials and methods

### 2.1. Study area

Theodore Roosevelt National Park is located in southwestern North Dakota, USA (45°55'N/103°31'W). The South Unit of the park, where this study was conducted, comprised approximately 19,000 ha and consisted of eroded badlands with gullies and ravines separated by large upland plateaus, and small buttes. The mixed-grass prairie vegetation was predominantly comprised of needle and thread (*Hesperostipa comata*), threadleaf sedge (*Carex filifolia*), western wheatgrass (*Pascopyrum smithia*), blue grama (*Bouteloua gracilis*) and little bluestem (*Andropogon scoparius*) (Butler et al., 1986). Sympatric ungulates included feral horses, pronghorn (*Antilocapra americana*), bison, mule deer (*Odocoileus hemionus*) and elk. Feral horses were confined to the South Unit by a 1.8–2.4 m high woven wire boundary fence and no other feral horse populations were nearby. All individual horses in this population were previously identified and assigned individual identity numbers by managers, and age and reproductive data for each animal have been maintained since 1993 (National Park Service, unpublished data).

### 2.2. Treatments and study subjects

In spring/summer 2009, when pre-treatment data were collected, 165 horses occupied the park and were organized into 19 bands (Table 1). All bands contained a single adult stallion and adult females and their offspring, with the

exception of one band that also contained a second stallion. No females persisted outside of bands; whereas, bachelor males were distributed across the landscape individually or sometimes in small ephemeral bands.

Horses were gathered into permanent corrals and handling facilities using a helicopter during a scheduled management capture in October 2009. Managers attempted to gather all horses in the population at that time, and removed 78 horses based on the agency's specific management needs. Eighty-one percent of the culled animals were foals, yearlings, and 2- and 3-year olds, and 14% were bachelor males not associated with any band. The remaining individuals removed included three females age  $\geq 16$  years, and one 6-year old female; none of which were part of our experimental group. These removals inherently skewed the sex ratio of the population between years because so many bachelor males were removed; however, paternity attributed to bachelor males is thought to be low (Bowling and Touchberry, 1990) and all study bands were subjected to competition pressure equally in each year at the population level. Post-treatment data were collected in spring/summer 2010, when 116 horses (including those born in 2010) occupied the park (Table 1).

We selected 58 adult females (2–17 years of age) using a randomized complete block design consisting of a GnRH-vaccinated treatment group and a saline control group. The resulting experimental female population consisted of 29 GnRH-vaccinated females (treatment) and 28 saline-treated (control) females, with one mature female remaining in the population that could not be captured. Females that were not in either treatment group were excluded from behavior analyses. The GnRH vaccine ultimately did not prevent conception in all inoculated horses (Baker et al., 2013); therefore, to more clearly separate the effects of infertility on behavior, we also excluded from analysis observations of 14 treatment females that became pregnant in 2010 and produced offspring in 2011. Females of both treatment groups were dispersed randomly across 16 bands with no difference in proportion of treated and control females between bands (paired *t*-test for unequal means:  $t=0.116$ ,  $P=0.909$ ). The same adults and bands observed in 2009 were observed in 2010, with none of the research cohort or band stallions removed during the culling operation.

Veterinarians blinded to treatment status assessed the general health, pregnancy, and body condition of each

animal at the time of capture. Pregnancy status and approximate stage of gestation were determined using rectal palpation of the reproductive tract and transrectal ultrasound imaging (Bucca et al., 2005). We visually assessed body condition of all females and scored condition using a numerical index that increased 1–9 with increasing fatness (Henneke et al., 1983). Females in the treatment group received an intramuscular injection in the gluteus muscle, by hand-syringe, containing 2000 µg of GnRH vaccine (2 mL, GonaCon-B™ (National Wildlife Research Center, Fort Collins, CO, USA)). Females in the control group were injected in a similar manner with an equal volume of saline solution. All data for this study were collected in accordance with Colorado State University Animal Care and Use Committee standards (protocol 12-3197A).

### 2.3. Behavioral sampling

Behavioral observations were conducted during three daylight time periods (08:00–12:00 h, 12:01–16:00 h, and 16:01–20:00 h) from April 18, 2009–July 16, 2009 and April 8, 2010–August 19, 2010. Each observation session included collection of a 20 min instantaneous scan sample of time budgets at 1 min intervals for each adult band member ( $\geq 1$  years old), and all-occurrence data collection for social interactions, exactly as implemented by a synonymous study of PZP in horses (Ransom et al., 2010). We used a feral horse ethogram that categorized specific behaviors into 15 discrete time budget categories; agonism, comfort, elimination, feeding, grooming, harem-social (e.g., allogrooming, pair-bonding, female-female urine marking), harem-tending (e.g. stallion defense of a band female or recruitment of a new female into the band), herding (e.g., driving or snaking behavior by the stallion), interaction-with-humans, locomotion, out-of-sight, reproduction, resting, standing attentive, and submission (Ransom and Cade, 2009). The infrequency and similar nature of several behaviors in the instantaneous scan samples led us to aggregate them into broader categories for time budget analyses. Grooming, comfort, standing attentive, and elimination were combined into *maintenance*; reproductive, harem-tending, harem-social, agonism, submission, and herding were combined into *social*; and interaction-with-humans and out-of-sight were combined into *unknown*. The time spent in the category *unknown* ultimately did not differ between treatment groups or between years.

We also collected social behavior data at every occurrence throughout each observation session because instantaneous scan sampling has the potential to underestimate rare events (Ransom and Cade, 2009). These all-occurrence behaviors included intraband social behaviors associated with herding, reproduction, agonism, and harem-tending and were retained as discrete categories for social behavior analyses. We observed all horses from the nearest distance that did not elicit attention of horses to the presence of the observer, typically 50–300 m. All observations were conducted using a 15–45 × 600 mm spotting scope or 10 × 42 mm binoculars when the distance

between horses and observers was too far to allow unassisted detailed observation.

### 2.4. Statistical analyses

We modeled the frequency of each behavior using mixed-effects linear regression, where individual female identity and sampling time period (time of day) were included as random effects on the intercept term of each model. This accounted for variation that may have been present among individuals who were sampled repeatedly, though not always equally over time, and for temporal variation in behavior when samples were not equally collected across all times of day. We used the lme4 package of R version 2.14.1 (The R Foundation for Statistical Computing 2011) and SYSTAT 12.02.00 (SYSTAT Software, Inc. 2007) to calculate descriptive statistics and obtain mixed-effects model estimates using restricted maximum likelihood (Harville, 1977).

We fitted separate models for each year to assess the fixed effects of treatment ( $x_1$ , pre-treatment or pre-control in 2009, treatment or control in 2010), foal presence ( $x_2$ , dependent foal < 1 year of age present with the female, or no foal present with the female), female age ( $x_3$ ), band size ( $x_4$ , total number of animals in the group), and band fidelity ( $x_5$ , number of times a female changed bands within the sampling year) on time budget behaviors. In the notation of Gelman and Hill (2007), models took the form of  $y_{jk[i]} = \alpha_{0j[i]} + \alpha_{1k[i]} + \beta_0 + \beta_{x_1}x_{1[i]} + \beta_{x_2}x_{2[i]} + \beta_{x_3}x_{3[i]} + \beta_{x_4}x_{4[i]} + \beta_{x_5}x_{5[i]} + \varepsilon_{jk[i]}$ , where the random effect intercept of  $\alpha_{0j[i]} \sim N(\mu = 0, \sigma_j^2)$  represents time period  $j$  (08:00–12:00 h, 12:01–16:00 h, 16:01–20:00 h), for  $i = 1, \dots, n$  observations, and the random effect intercept  $\alpha_{1k[i]} \sim N(\mu = 0, \sigma_k^2)$  represents  $k = 1, \dots, n$  individual horses for  $i = 1, \dots, n$  observations. This equation identifies the fixed effect intercept as  $\beta_0$ ,  $x$  = independent variable with parameter  $\beta_x$ ,  $\varepsilon$  = statistical error, and  $y$  = dependent variable. All-occurrence social behavior was modeled in the same framework, but because those data involved a given stallion directing a behavior toward a given female, we also included the additional variable of stallion age as a fixed effect.

In order to gain insight into the effects of removal versus fertility control, we also modeled time budget and all occurrence behavior across both years, replacing treatment status with observation year as a fixed effect. This allowed us to look at the broad paradigm change, independent of trying to separate the inherently related aspects of changing sex ratio, density, and competition. We included Normalized Difference Vegetation Index (NDVI) as a fixed effect in this model in order to estimate the temporal influence of forage availability on behavior that could have varied between years. The NDVI data were obtained from the National Aeronautics and Space Administration (<http://modis.gsfc.nasa.gov>) and reconciled using ArcGIS software (ESRI, Redlands, California). We also included sex as a fixed effect in this model to gain insight into both male and female time budgets in concert with the culling perturbation.

### 3. Results

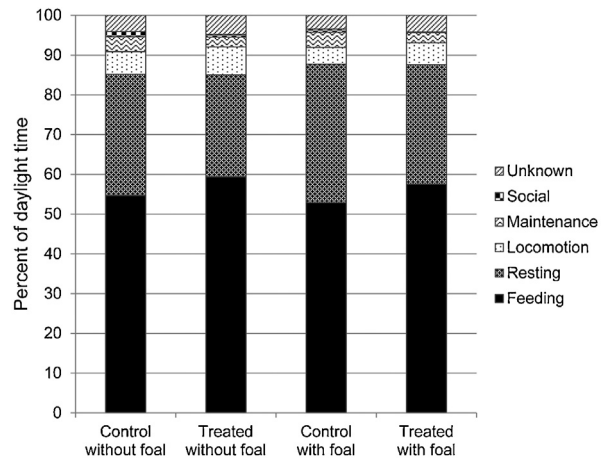
The 2009 pregnancy assessment indicated that 89% of the control group and 86% of the treatment group were pregnant at the time of saline- or GnRH vaccine-injection. In the following year, 71% (95% CI = 53.9–89.3%) of control females and 72% (95% CI = 55.1–89.7) of treated females were observed with a dependent offspring. Mean body condition of pre-control females was 5.1 (95% CI = 4.8–5.4) and of pre-treatment females was 4.9 (95% CI = 4.7–5.2) in 2009. Likewise, mean body condition of control females was 4.9 (95% CI = 4.7–5.1) and of treated females was 4.8 (95% CI = 4.7–4.9) in 2010.

We collected behavioral data on 93 feral horses (23 males, 29 pre-treatment females, 27 pre-control females, 14 other females) for 582.4 h in 2009. The median age of observed stallions was 7 years (range = 3–14 years), median age of observed females was 5 years (range = 2–17), and median band size was 8 horses (range = 3–17). In 2010, approximately 5 months after treatment and culling, we observed 77 of the same feral horses (18 males, 29 treated females, 28 control females, two other females) for 1263.90 h. The median age of observed stallions was 9 years old (range 5–15), median age of observed females was 5 years old (range = 3–18), and median band size was eight horses (range = 3–19). The final data used for GnRH treatment analyses arose from 1331 observations of 29 pre-treated and 29-pre-control females in 2009 and 2299 observations of 15 treated and 28 control females in 2010. The final data used for culling analyses arose from 2115 observations of 23 males, 27 pre-control females and 29 pre-treatment females in 2009, and 3274 observations of 18 males, 28 control and 29 treatment females in 2010.

#### 3.1. Time budgets

Band size and foal presence were influential in how females allocated their time during 2009, but there were no differences in behaviors between pre-treatment and pre-control females (Table 2). However, treated and control females spent their time differently in the year following inoculation. Treated females fed more and moved more than control females, and reciprocated by reducing time in resting and maintenance behaviors (Fig. 1). There were no changes in time spent engaging in social behaviors between treated or control females and their conspecifics (Table 2). Band size influenced feeding time, increasing  $0.9 \pm 0.40$  (SE)% with each additional horse in the band in 2009. This effect was not evident in 2010 after the population was reduced. Time of day influenced feeding behavior in both years. Horses fed an estimated  $7.3 \pm 4.69\%$  (2009) and  $7.5 \pm 5.01\%$  (2010) less in the morning, as compared to  $7.4 \pm 5.00\%$  (2009) and  $9.0 \pm 5.04\%$  (2010) more in the afternoon.

Time spent feeding was largely reciprocated by time resting among both treated and control groups (Fig. 1). There were no differences between pre-treatment and pre-control females in time spent resting ( $t_{1270} = 0.459$ ,  $P = 0.646$ ), but like feeding, this relationship changed after inoculation, and treated females rested less than their control counterparts (Table 2). Foal presence influenced



**Fig. 1.** Mean allocations of daylight time budget behaviors for feral horse (*Equus caballus*) females at median age (5 years old) and median band size (eight horses). Estimates are shown from mixed-effects linear regression considering individual identity and time of day as random effects. Data consisted of 2299 observations of 28 control and 15 treatment females in 2010 at Theodore Roosevelt National Park, USA. Foal presence was defined as a dependent born during the observation year.

resting in both years with independent females resting  $4.4 \pm 2.27\%$  (2009) and  $4.4 \pm 1.83\%$  (2010) less than females with a dependent foal (Table 2). Resting occurred temporally opposite of feeding activity, with  $7.0 \pm 4.43\%$  (2009) and  $6.5 \pm 4.18\%$  (2010) more resting in the morning and  $6.8 \pm 4.72\%$  (2009) and  $7.2 \pm 4.22\%$  (2010) less in the afternoon.

The remainder of horses' time was occupied by locomotion (about 5% of their time), maintenance (about 3% of their time), and social behavior (about 1% of their time) (Fig. 1). Treated females spent less time moving than controls and reciprocated with increased maintenance behavior (Table 2). Age was a statistically significant effect in modeling locomotion in 2010, but because time in locomotion only differed by 0.12% per year of age, this is unlikely to be biologically significant. Foal presence, however, was a strongly supported effect and females with a dependent were on the move  $1.5 \pm 0.35\%$  more than independent females (Table 2). Maintenance behavior was influenced by band fidelity in 2010 and time spent exhibiting this behavior increased  $1.6 \pm 0.44\%$  per band change. Six of 43 females (14%) were observed changing bands one to three times during 2010. In contrast, 23 of 58 females (40%) changed bands one to four times in 2009. The decreased band size in 2010 appears to have influenced time spent performing maintenance behavior (Table 2). Like age and locomotion, this effect was statistically significant but with an increase of only  $0.1 \pm 0.06\%$  per additional band member it is unlikely to have biological meaning. Locomotion, maintenance, and social time budget behaviors were influenced minimally by time of day or individual variation (Table 2).

#### 3.2. Social behaviors

All-occurrence social behaviors associated with herding, harem-tending, reproduction, and agonism from stallions toward females were observed 57% of the time

**Table 2**

Supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) time budget behaviors at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day ( $j$ ) and individual horse identity ( $k$ ) are shown as  $\sigma_j^2$  and  $\sigma_k^2$ .

Behavior	Supported effect	$t$	$P$	Difference	95% lower confidence limit	95% upper confidence limit	$\sigma_j^2$	$\sigma_k^2$
2009								
Feeding	Band size	2.217	0.027	0.878	0.101	1.654	0.589	0.163
Resting	Foal presence	-1.923	0.054	-4.370	-8.828	0.089	0.524	0.156
Locomotion	Band size	-2.369	0.018	-0.186	-0.340	-0.032	<0.001	0.009
Maintenance	None	-	-	-	-	-	0.007	0.100
Social	Foal presence	1.936	0.053	0.183	-0.002	0.369	<0.001	<0.001
2010								
Feeding	Treatment	-2.064	0.039	-4.685	-9.136	-0.234	0.716	0.188
Resting	Treatment	2.239	0.020	4.826	0.762	8.890	0.490	0.125
	Foal presence	-2.420	0.016	-4.425	-8.010	-0.840		
Locomotion	Treatment	-3.511	<0.001	-1.319	-2.056	-0.582	0.002	<0.001
	Age	2.059	0.040	0.117	0.005	0.229		
	Foal presence	4.304	<0.001	1.522	0.829	2.217		
Maintenance	Treatment	2.655	0.008	1.266	0.330	2.200	0.006	<0.001
	Band size	2.449	0.014	0.136	0.027	0.245		
	Band fidelity	3.617	<0.001	1.574	0.721	2.430		
Social	None	-	-	-	-	-	0.002	0.013

( $n=331$ ) during 2009 and 51% of the time ( $n=650$ ) during 2010. Herding by stallions was the most frequently observed social behavior. Band size influenced this behavior in 2009, with herding increasing  $0.16 \pm 0.56$  times per hour (tph) per additional horse in the band (Table 3). In 2010, however, age of the stallion was the only important fixed effect on this behavior and herding increased  $0.18 \pm 0.038$  tph per year of age. At median stallion age, median female age, and median band size, pre-treatment females received herding behavior  $2.24 \pm 0.743$  tph and pre-control females received this behavior  $1.98 \pm 0.694$  tph ( $t_{274} = -0.892$ ,  $P=0.373$ ); likewise, treated females received herding behavior  $1.06 \pm 0.415$  tph and control females received this behavior  $0.99 \pm 0.454$  tph ( $t_{602} = -0.271$ ,  $P=0.787$ ). Time of day was not an important influence on herding in either year and variance of this random effect was similar in both years (Table 3).

Harem-tending occurred less frequently than herding in 2009, but at a similar rate as herding in 2010. The supported fixed-effects in models of harem-tending were band size in 2009 and age of stallion in 2010 (Table 3). As band size increased per horse, harem-tending decreased  $0.05 \pm 0.020$  tph in 2009, and as stallion age increased per

year, harem-tending decreased  $0.11 \pm 0.026$  tph in 2010. As with herding behavior, time of day did not influence harem-tending occurrence in either year and there was little individual variance in 2009.

Contrary to our prediction that reproductive behaviors would be reduced in GnRH-treated mares compared to controls, we observed almost exactly the same frequency in both years and between treatment groups. At median stallion age, median female age, and median band size pre-treatment females received reproductive behaviors  $0.57 \pm 0.374$  tph and pre-control females received reproductive behaviors  $0.40 \pm 0.349$  tph ( $t_{274} = -1.054$ ,  $P=0.293$ ); likewise, treatment females received reproductive behaviors  $0.59 \pm 0.270$  tph and control females received reproductive behaviors  $0.56 \pm 0.297$  tph ( $t_{602} = -0.177$ ,  $P=0.860$ ). The only supported fixed-effect in either year was female age in 2009, which estimated a  $0.05 \pm 0.024$  tph decrease in reproductive behavior per year of age.

Agonism occurred at similar rates as reproductive behaviors in both years and was influenced by stallion age in both years (Table 3). Agonism from males toward females increased  $0.03 \pm 0.019$  tph per year of male age in 2009 and  $0.04 \pm 0.017$  tph per year of male age in 2010. At

**Table 3**

Supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) social behavior at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day ( $j$ ) and individual horse identity ( $k$ ) are shown as  $\sigma_j^2$  and  $\sigma_k^2$ .

Behavior	Supported effect	$t$	$P$	Difference	95% lower confidence limit	95% upper confidence limit	$\sigma_j^2$	$\sigma_k^2$
2009								
Herding	Band size	2.871	0.004	0.159	0.050	0.267	0.072	<0.001
Harem-tending	Band size	-2.731	0.007	0.054	-0.093	-0.015	<0.001	<0.001
Reproduction	Female age	-2.094	0.037	0.051	-0.099	-0.003	<0.001	0.073
Agonism	Stallion age	1.990	0.048	0.038	0.000	0.076	<0.001	<0.001
2010								
Herding	Stallion age	4.906	<0.001	0.184	0.110	0.258	0.047	0.153
Harem-tending	Stallion age	-4.234	<0.001	0.110	-0.162	-0.059	0.007	0.163
Reproduction	None	-	-	-	-	-	<0.001	0.096
Agonism	Stallion age	2.436	0.015	0.042	0.008	0.076	0.026	<0.001
	Band fidelity	2.411	0.016	0.201	0.037	0.365		

median stallion age, median female age, and median band size pre-treatment females received agonistic behaviors  $0.26 \pm 0.251$  tph and pre-control female received agonistic behaviors  $0.44 \pm 0.234$  tph ( $t_{274} = 1.744$ ,  $P = 0.082$ ); likewise, treatment females received agonistic behaviors  $0.19 \pm 0.184$  tph and control females received agonistic behaviors  $0.36 \pm 0.203$  tph ( $t_{602} = 1.627$ ,  $P = 0.104$ ). Band fidelity also influenced the 2010 estimate and agonism increased with number of band changes (Table 3). Neither time of day nor individual variance influenced agonism occurrence in either year.

### 3.3. Population reduction

Horses allocated time differently by sex and by year (Table 4). Males fed less, rested more, moved more and engaged in social interactions more often than females, but all horses fed less, rested more, moved more, and performed less maintenance behavior and engaged in more social behavior in 2010 as compared to 2009 (Fig. 2). Normalized Difference Vegetation Index was highly correlated between years ( $R^2 = 0.90$ ). The finer scale all-occurrence behavioral data revealed that herding by stallions decreased 50.71% from 2009 to 2010, and appeared to increase in frequency concurrent with the seasonal green-up period (and concurrent reproductive season) (Fig. 3A). Harem-tending behavior increased 195.0% from 2009 to 2010, when both were estimated at median stallion age, median female age, median band size, and mean NDVI. Harem-tending also occurred in concert with forage production, but this relationship was more pronounced in 2010, when the frequency of occurrence was markedly escalated (Fig. 3B). Time of day and individual identity contributed little to variance in these models (Table 4).

## 4. Discussion

There were no differences in any behaviors between treatment groups before GnRH vaccination and culling. Females vaccinated against GnRH managed their time differently than controls. They fed less, rested more, traveled less, and performed more maintenance behaviors than their control counterparts, but engaged in the same amount of social behavior. The estimates across years, however, indicated that pronounced behavioral changes occurred in how stallions engaged with females in social behaviors. Stallions herded all females less and harem-tended more following the changes in population density, demography, and social perturbation that resulted from culling. The date of observation in relation to forage availability explained some differences between years, while the increased mean age of stallions as well as the decreased band size in 2010 also explained some of the differences. None of the models indicated that herding or harem-tending varied due to the treatment status of females, but rather implied that the paradigm shift in competition influenced how band stallions managed their family groups. Because the same stallions were observed in both years, the effect of age potentially reflected the additional year of experience each male possessed, in the context of competition pressure.

The similarity in female social behavior between treatment groups likely related to estrous behavior, a catalyst of other social behavior. Estrous behavior is influenced by the concentration and timing of progesterone and estradiol, the steroid hormones that fluctuate with occurrence of pregnancy (Katz, 2007). During pregnancy, high concentrations of progesterone usually inhibit estrous behavior (Crowell-Davis, 2007; Fabre-Nys and Gelez, 2007). The GnRH-antibodies stimulated by GnRH vaccination should have interrupted the hormonal cascade in females and ultimately suppressed pulsatile luteinizing hormone (LH) secretion, and thus ovulation (Powers et al., 2011). Consequently, we might expect that similar social behavioral expressions (albeit for different reasons) between pregnant females and GnRH-treated females would be observed. However, a similar study in elk concluded that GnRH was not completely suppressed in vaccinated females and thus some LH stimulation occurred and likely allowed limited production of estradiol (Powers et al., 2011). This permitted reproductive behaviors, at a reduced level, in GnRH-treated individuals. It should also be noted that estrous behavior has been observed with low frequency among both pregnant female and anovulatory female horses (Asa et al., 1983; Crowell-Davis, 2007). This is thought to be a bonding mechanism that assists in the maintenance of stable social groups of horses year-round, rather than ephemeral reproductive groups, such as those observed in elk. Also, the complex social behaviors among feral horses do not entirely center on reproductive receptivity, and other behaviors associated with dominance hierarchies, leadership, and gregarious lifestyles may or may not be as directly influenced by fluctuations in progesterone and estradiol.

Regardless of the similarity in social behaviors between treatment groups, treated females still allocated their time budgets differently than controls in 2010. Most GnRH-vaccinated females in the final dataset were likely not pregnant in 2010 because we categorically omitted the treated females that demonstrated unsuccessful response to vaccination by producing offspring in 2011. Pregnancy influences time spent feeding and resting simply due to the metabolic needs of barren females as compared to pregnant females (National Research Council, 2007). The composite nature of time budget behaviors requires that if any one behavior changes, then other behaviors such as locomotion or maintenance must also change; thus, the small differences between treatment groups were unsurprising.

The estimates of time spent per behavior for all horses in our study fell well within the ranges previously reported for daylight time budgets of horses (Mayes and Duncan, 1986; Boyd, 1998; Berger et al., 1999; Ransom et al., 2010). Ultimately, body condition scores of control and treated females were at parity in 2010. This may indicate that the metabolic demands of pregnant and non-pregnant females were compensated for behaviorally, that the body condition scoring system was not sensitive enough to detect finer-scale differences, or that forage abundance was high enough to obfuscate the effects of behavior on body condition. Foal presence influenced resting in both years with independent females resting less than females with a

**Table 4**

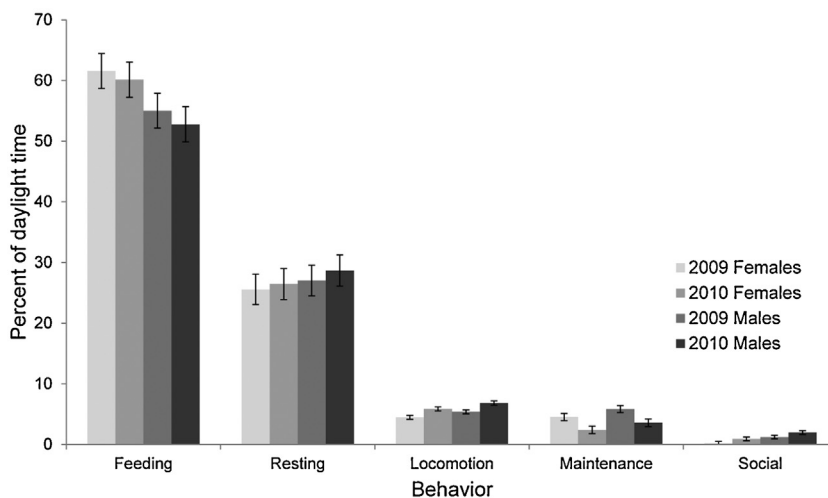
Between year differences in time budget and social behaviors of feral horses (*Equus caballus*) Theodore Roosevelt National Park, USA, as estimated by mixed-effects linear regression. Variance for the random effects of time of day ( $j$ ) and individual horse identity ( $k$ ) are shown as  $\sigma_j^2$  and  $\sigma_k^2$ .

Behavior	Supported effect	$t$	$P$	Difference	95% lower confidence limit	95% upper confidence limit	$\sigma_j^2$	$\sigma_k^2$			
Time budget	Feeding	Year	2.141	0.032	2.141	1.838	4.178	0.743	0.098		
		Sex	5.413	<0.001	6.725	4.289	9.160				
		Band size	3.699	<0.001	0.497	0.234	0.761				
		Age	-3.040	0.002	-0.477	-0.784	-0.169				
	Resting	NDVI	-22.407	<0.001	-115.698	-125.820	-105.576				
		Age	2.844	0.004	0.445	0.138	0.752			0.515	0.104
	Locomotion	NDVI	19.903	<0.001	100.605	90.695	110.514				
		Year	-5.726	<0.001	-1.280	-1.719	-0.842			0.001	<0.001
		Sex	-4.014	<0.001	-0.917	-1.366	-0.469				
	Maintenance	Band size	-3.720	<0.001	-0.098	-0.150	-0.046				
		NDVI	-5.433	<0.001	-6.307	-8.582	-4.031				
		Year	5.122	<0.001	1.754	1.083	2.425			0.014	0.014
	Social	Sex	-2.957	0.003	-1.279	-2.127	-0.431				
NDVI		15.987	<0.001	27.654	24.263	31.045					
Year		-3.862	<0.001	-0.714	-1.077	-0.352	0.001	0.014			
Sex		-3.095	0.002	-0.991	-1.618	-0.363					
All occurrence	Band size	2.013	0.044	0.057	0.002	0.113					
	NDVI	6.798	<0.001	0.062	0.044	0.079					
	Herding	Year	7.581	<0.001	1.148	0.851	1.445	<0.001	0.063		
		Stallion age	5.226	<0.001	0.026	0.088	0.193				
Harem-tending	Year	-9.483	<0.001	-0.838	-1.012	-0.665	0.004	0.970			
	Stallion age	-4.578	<0.001	-0.078	-0.111	-0.044					
	Band size	-4.793	<0.001	-0.057	-0.080	-0.033					
	NDVI	4.918	<0.001	2.385	1.433	3.336					
Reproduction	Band size	-2.242	0.025	-0.025	-0.047	-0.003	<0.001	0.044			
	NDVI	2.095	0.037	1.019	0.065	1.973					
Agonism	Stallion age	2.938	0.003	0.035	0.012	0.058	0.009	0.008			
	NDVI	-2.736	0.006	-1.032	-1.772	-0.292					

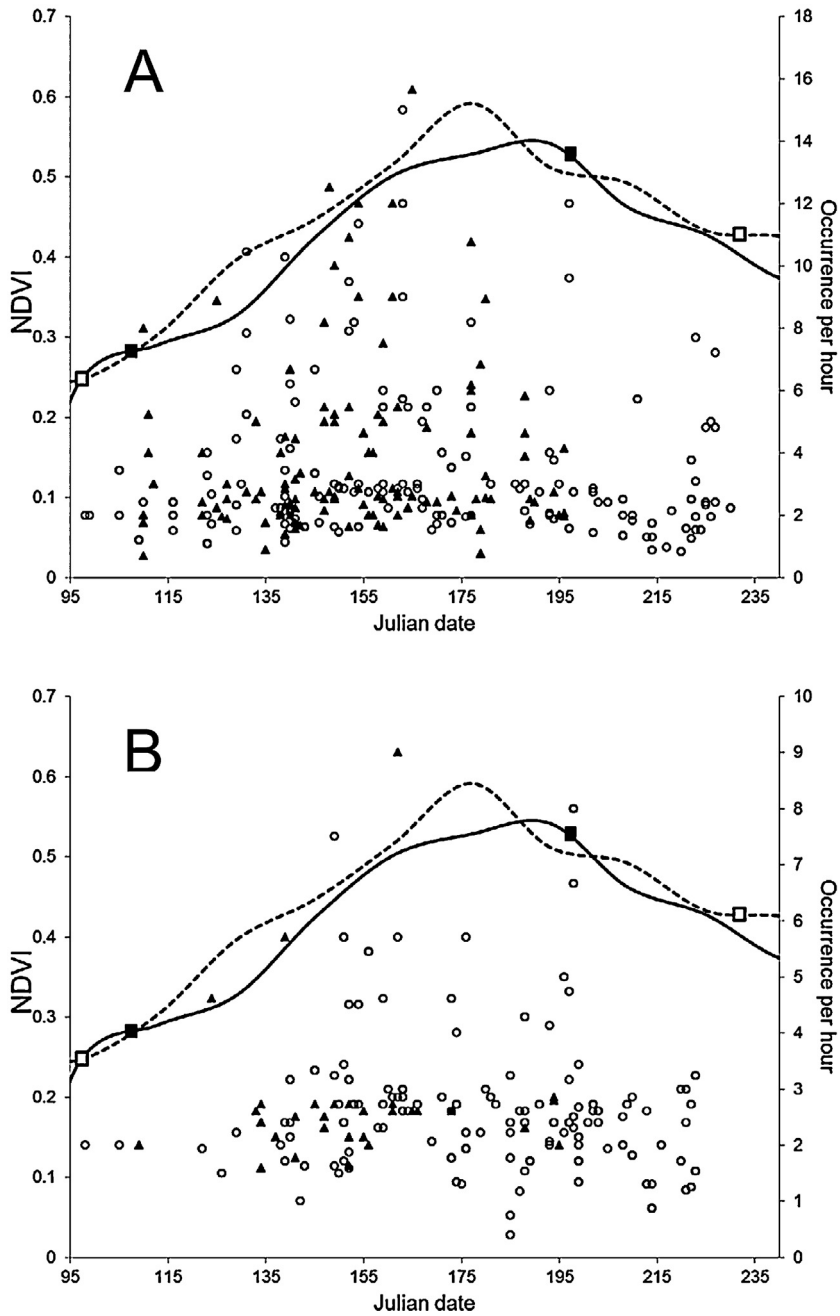
dependent foal. Band size was also an important effect on some behaviors; most notably, herding and harem-tending behavior in 2009 were significantly related to band size, but this was not the case in 2010. This effect on social behaviors was replaced with stallion age in 2010. These contradictions may be in part due to the change in population size that occurred between years or due to changes in

band composition. Even though band size did not change notably between years, some young animals were removed from bands.

Density of bachelor males in the population was likely influential in the large changes in herding and harem-tending behaviors observed between years: the number of bands and band size did not differ greatly between



**Fig. 2.** Comparison of mean  $\pm$  SE daylight time budget behaviors of feral horses (*Equus caballus*) before (2009) and after (2010) culling and GnRH treatment at Theodore Roosevelt National Park, USA. Estimates are shown at median age, median band size, and mean Normalized Difference Vegetation Index (NDVI) and arose from mixed-effects linear regressions considering individual identity and time of day as random effects.



**Fig. 3.** Temporal occurrence and rate of feral horse (*Equus caballus*) herding (A) and harem-tending (B) behaviors in relation to seasonal trend in forage availability using Normalized Difference Vegetation Index (NDVI) as an index of greenness. NDVI for the pre-treatment year of 2009 is shown as a solid line with behaviors as solid triangles and NDVI for the post-treatment year of 2010 is shown as a dashed line with behaviors as hollow circles. Boxes on the NDVI trend lines designate the beginning and end of each behavior observation season.

years, but the number of available males in the population decreased 41% between years and the mean age of males in the population increased by nearly 3 years or 36%. This combination of effects presents compelling evidence that quickly and non-randomly reducing population density has behavioral implications for individuals and how bands function in the social dynamics of the population.

Herding and harem-tending behavior are important behaviors used by band stallions to maintain bands, but also represent an energetic cost for both the male and female(s) involved. Such costs may have biological meaning in terms of body condition and fidelity of females to the band (Ransom et al., 2010). The decline in herding behavior detected between years is not surprising given the decreased presence of competing males in the



population, which is often what prompts a stallion to actively herd females together (Waring, 2003). This decrease in potential energetic expense lends support for the practice of culling because while it decreases grazing impact by removing animals, it also leads to the remaining animals expending less energy (that would have to be replaced by grazing) by decreasing frequency of behaviors that arise from competition. Consequently, 2.2% less feeding behavior was estimated for stallions in 2010 as compared to 2009 in our study. This is supported by the observed increase in harem-tending behaviors in our study, most of which are likely driven by instinctual behavioral mechanisms more than active presence of a competitor. Such behaviors include stallions marking urine deposits of band members and constructing fecal middens that relate to reproductive status, communication, and band identification (Feist and McCullough, 1976; Rubenstein and Hack, 1992).

Little has been studied on the effects of culling in feral horse populations, and what has been presented in the literature focuses on stress and its effects on reproduction (Hansen and Mosley, 2000; Ashley and Holcombe, 2001). A short term study did collect data on percent of time horses spent feeding, resting, vigilant, traveling, and engaged in agonistic encounters and found no difference between control (horses not gathered or pursued) and simulated (horses that were pursued but evaded capture) groups (Hansen and Mosley, 2000). That study did not investigate the broader effects relating to density and sex ratio changes. We could find no other studies that investigated behavior before and after population removals of feral horses. There are some comparable studies of feral horse behavior in relation to immunocontraception however.

Porcine zona pellucida is the most extensively investigated immunocontraceptive for feral horses, and recent studies have found PZP-treated females exhibit more frequent reproductive behaviors and seasonally prolonged reproductive behaviors than their control counterparts. In a comparable study of three feral horse populations, 54.5% more reproductive behaviors were received by PZP-treated females than by control animals during the breeding season (Ransom et al., 2010). Likewise, in another feral horse population, more frequent reproductive behaviors among PZP-treated females were observed in the non-breeding season (Nuñez et al., 2010). Elevated rates of estrus have been reported in PZP-treated females of other species as compared to control females, which likely explains this increase in observed reproductive behavior (Mahi-Brown et al., 1985; Shumake and Wilhelm, 1995; Heilmann et al., 1998; Curtis et al., 2002). Seasonality of estrus in horses may not be as constrained by abiotic factors as previously thought, so it is possible that any contraceptive that does not completely suppress estrus could be associated with seasonally atypical expressions of reproductive behavior (Ransom et al., 2013). Decreased band fidelity has also been reported among PZP-treated female horses (Nuñez et al., 2009; Madosky et al., 2010), and in our GnRH study the per capita female interchange rate decreased 25.7% from 2009 to 2010. Given that some social reorganization was expected following the culling perturbation, the observed decrease in interchange provides additional evidence

that GnRH-vaccinated females were not expressing the frequent reproductive behavior that appears to be coincident with decreased band fidelity.

Studies of the effects of GnRH immunocontraceptive vaccines on behavior of female horses are limited; nevertheless, a few small studies do illustrate some general patterns in responsiveness. In the only other feral horse study reported in the literature, no differences were observed between treatment groups in time spent feeding, resting, or traveling in either the breeding or non-breeding season (Gray et al., 2010). Of the 25 copulations observed in that study, only one was a GnRH-vaccinated female and it occurred during the non-breeding season. Both treated and control females demonstrated reproductive behavior roughly once every 2 h in our study. Our results concur with observations of reproductive behaviors in controlled experiments where captive mares were treated with GnRH vaccine. In those studies, treated mares showed suppressed ovarian activity and ovulation after immunization but continued to show evidence of estrous behavior at irregular intervals and duration (Dalin et al., 2002; Imboden et al., 2006). Similar behavioral responses to contraception induced by GnRH vaccine and GnRH agonist have been reported in other ungulate species as well (Baker et al., 2002, 2004; Conner et al., 2007; Powers et al., 2011). The parity we observed between treatment groups therefore simply reflects the limited reproductive behavior among control females due to high rates of pregnancy and suppressed reproductive behavior among treated females due to GnRH-inhibited ovarian activity. Since GnRH is highly conserved across mammalian species, fertility control technology founded on this hormone may suppress reproduction but does not always prevent the occurrence of sexual behaviors.

Our study was limited to only a pre-treatment and treatment year, and as such inferences should only be extended to the individual level in the short term. Both pregnancy and birth rates between treatment groups in our study were the same, but variation in birth interval among individuals can make longer term trends less certain in terms of births, dependent foals, physiology, and associated behaviors. While some studies have demonstrated females may be less likely to foal in consecutive years, as opposed to alternate years, others have found no differences (Roelle et al., 2010). Probability of foaling can also be strongly related to band size (Roelle et al., 2010), which was equitable between years in our study but may trend downward with effective fertility control over long time periods. For these reasons, the short duration of our study provides some relatively clear inference toward short-term effects of GnRH and culling on individual behaviors; however, long term effects of fertility control on behavior may manifest to all individuals as a population paradigm shifts to lower fecundity, and variable and unknown effects on social structure and dynamics emerge.

## 5. Conclusion

Our study found only minimal differences in behavior of free-ranging female feral horses treated with GnRH

and those treated with saline. Those differences could be physiologically explained and body condition between the two treatment groups was at parity. Following the same methodological protocols as a similar study of PZP-treatment on behavior of feral horses (Ransom et al., 2010), GnRH appears to lead to fewer behavioral modifications than PZP in this species in the short term. Long term influences of GnRH remain uncertain. The ultimate assessment of any fertility control agent must include the scope and magnitude of all potential contraindications, vaccine efficacy, and weighing the known effects against the benefit of reducing population growth. The differences in how band stallions socially interacted with all females between years provided strong evidence related to the effects of culling. These behavioral responses shed light on the effects of reducing competition and density in a social polygynous species with strong group fidelity. Such perturbation may be synergistic in reducing grazing pressure by decreasing energetically expensive competitive behaviors, but further investigation is needed to explicitly test this hypothesis.

### Conflict of interest

We declare no conflict of interest among financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, our work.

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**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.

A handwritten signature in black ink that reads "Sally Hall". The signature is written in a cursive, flowing style.

**Sally Hall**

Bachelor of Animal and Veterinary Bioscience (Honours)

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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.

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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.

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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.

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## 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



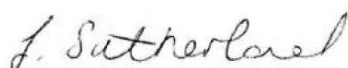
Doctor Zamira Gibb

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. 18<sup>th</sup> 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. 44<sup>th</sup> Annual Conference of the Society for Reproductive Biology, 25-28 August 2013, Sydney, Australia

#### **4. Additional co-authored publications**

**Hall SE**, Negus C, Johnke 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

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## **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 REVIEW**

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

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**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.

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

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**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.

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### 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 <i>et al.</i> (2007), Janett <i>et al.</i> (2009)
	Improvac	Established	Female	2	1–2 years	100	Imboden <i>et al.</i> (2006), Botha <i>et al.</i> (2008)
	GonaCon	Established	Male and female	1	1–4 years	40–94	Killian <i>et al.</i> (2006), Killian <i>et al.</i> (2008), Miller <i>et al.</i> (2008), Gray <i>et al.</i> (2010)
ZP	SpayVac	Established	Female	1	≥3 years	83–100	Powell and Monfort (2001), Killian <i>et al.</i> (2006), Killian <i>et al.</i> (2008), Turner <i>et al.</i> (2008), Gray <i>et al.</i> (2010), Bechert <i>et al.</i> (2013), Mask <i>et al.</i> (2015)
Proteins found on primordial follicles, spermatogonial stem cells, Sertoli cells	Phage peptides	Emerging	Male and female	1	Lifelong (intended)	–	Eidne <i>et al.</i> (2000), Meloen <i>et al.</i> (2000), Aitken (2006), Samoylova <i>et al.</i> (2010, 2012, 2012, 2015)
Proteins found on spermatozoa, oocyte and blastocyst	Aldehyde-adducted proteins	Future	Male and female	1	Lifelong (intended)	–	Dixit <i>et al.</i> (1975), Bohring and Krause (2003), Frank <i>et al.</i> (2005), Klein <i>et al.</i> (2010), Wang <i>et al.</i> (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.

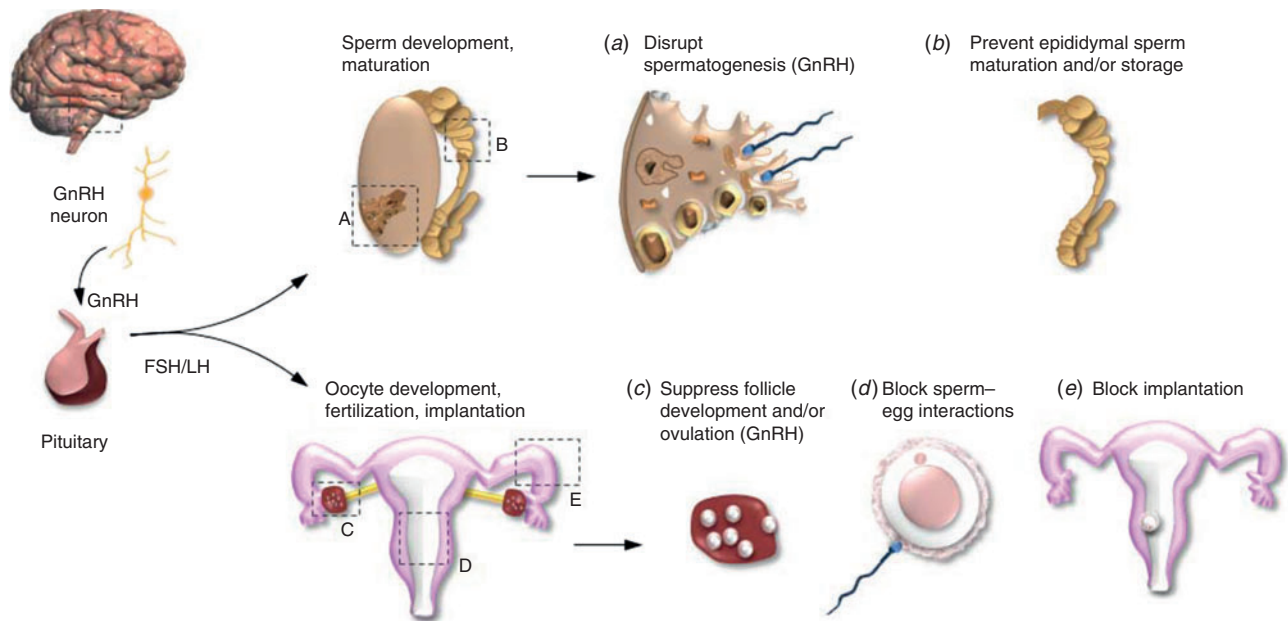
### Fertility control currently used for feral horse management

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 (Meloan *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 FSH $\beta$ –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 spermatogenesis and folliculogenesis 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–olemma 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 immunoneutralisation 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  $\alpha$ -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 trophoctoderm release large amounts of interferon- $\tau$  (IFNT), which suppresses prostaglandin (PG) F<sub>2 $\alpha$</sub>  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<sub>2 $\alpha$</sub>  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<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> 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.* 2014a). 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.* 2014b). 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.

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## **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**

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## 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.

# 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

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**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.

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## 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.* 2013a). 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; Francioli *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 diazobicyclo-[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 000g at room temperature and the supernatant transferred to a gDNA eliminator spin column and centrifuged for 30 s at 13 000g 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 8000g 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)
<i>GAPDH</i>	NM_001163856.1	TCAAGTCATTTCTGGTAT	CCTTCTCCTTCTCTTGCT	130	58/55
<i>PI3K</i>	XM_001497768.3	TGCAGCTTAAGATAGGAAGT	TCTTGGTACAGTCCTGAAAA	100	58
<i>AKT1</i>	XM_005605498.1	CAAACACCGTACCATGAAC	GATGACTTCCTTCTTCAGGA	133	55
<i>PTEN</i>	XM_006526769.2	ACAGCCATCATCAAAGAGATCGT	TCAAGTCTTCTGCAGGAAATCCCA	125	58
<i>JAK1</i>	XM_001918306.2	AACCACATAGCTGATCTGAA	AATACCATTTCTCCGTCTT	108	58
<i>STAT3</i>	XM_001493633.2	AAATGGGGATTTCTGAGGAT	TTCAGGAATATCTCAGGACG	127	58
<i>SOCS4</i>	NM_001256911.1	TCTCTGTTAGTTTtaggCGT	CACTGGGGCCTTATAGTGT	147	55

discarded and 600 µL Buffer RW1 added to the spin column before centrifuging for 15 s at 8000g 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 000g at room temperature. Buffer RPE (500 µL) was added to the spin column and centrifuged for 2 min at 8000g 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 8000g 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.* 2013b), 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.

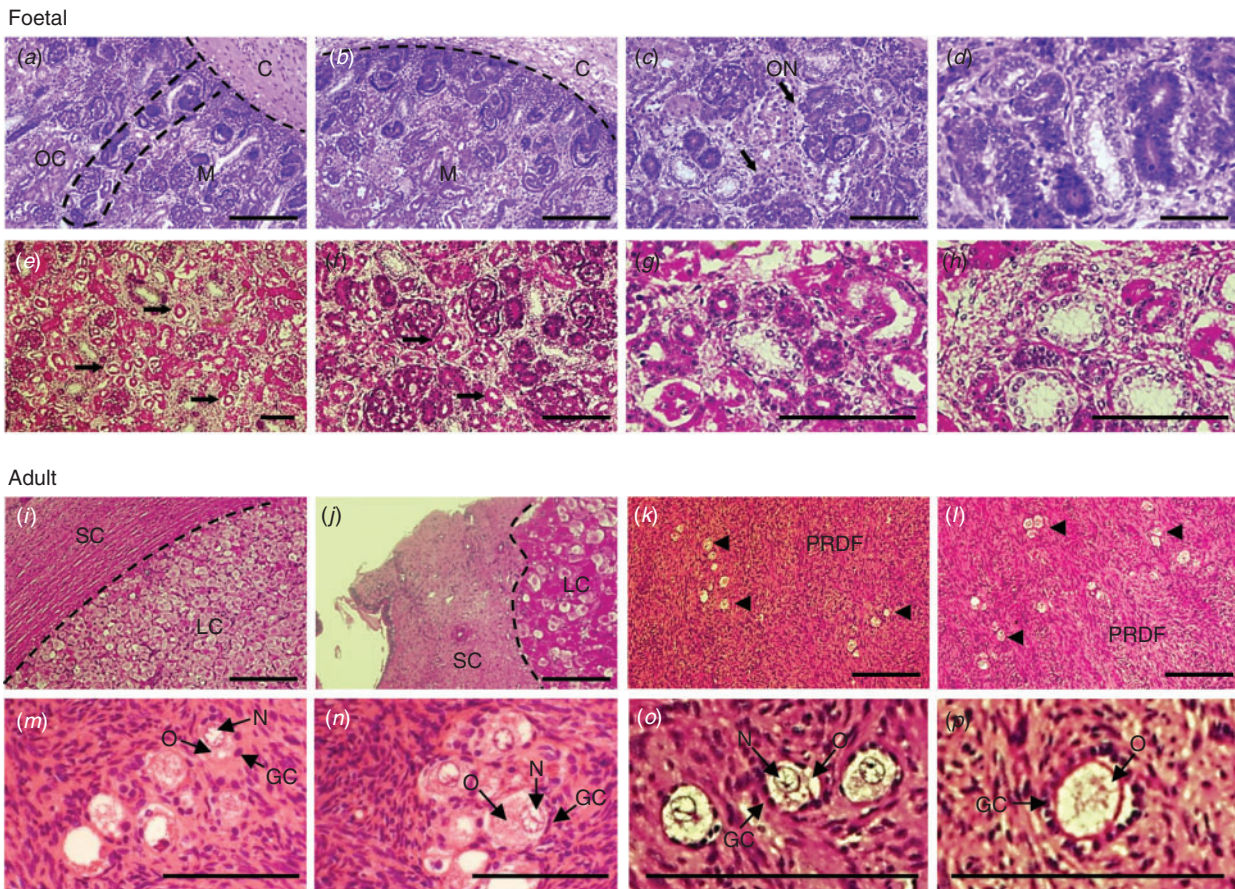
## Results

### 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. 1a–b). Ovigerous cords (OC) can be distinguished running down the medulla to the centre of the ovary (Fig. 1a). Oogonial nests (ON) and individual oogonia (O) are observed throughout the tissue (Fig. 1c, e–f), often surrounding a site of vascularity (Fig. 1d, g–h). In adult mare ovarian tissue, tissue is primarily composed of somatic cells (SC) and luteal cells (LC; Fig. 1i–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. 1k–o; McEntee 2012). Primordial follicles are differentiated from primary follicles (Fig. 1p) 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.* 2006b), was expressed in somatic cell streams undergoing DNA synthesis in the fetal ovary (Fig. 2a). 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. 2b). 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. 2c). 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 primarily 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  $\mu$ M.

(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. 2d). 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.

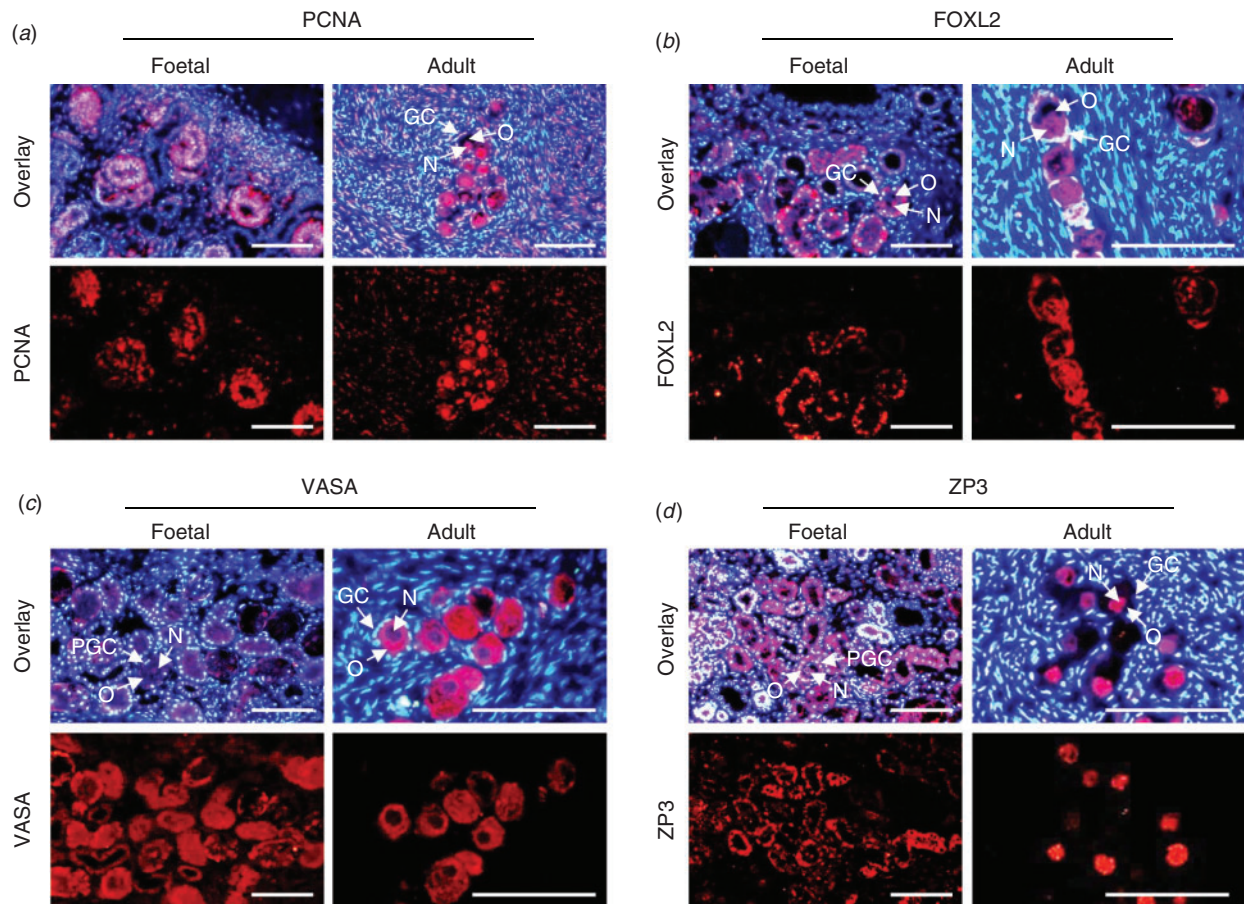
#### *PI3K/AKT mRNA expression is significantly higher in the adult mare ovary than in the fetal ovary*

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. 3a). *AKT1* mRNA expression increased in a similar manner

( $P < 0.01$ ; Fig. 3b). 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. 3c). 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. 3d–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  $\mu$ M.

cytoplasm of the oocyte in the adult ovary (Fig. 3d), 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. 3e). Finally, PTEN remained localised to the granulosa cells in the adult ovary (Fig. 3f).

#### *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. 4a). The trend of *STAT3* mRNA expression matched *JAK1*, with a significant increase in *STAT3* expression in the adult ovary ( $P < 0.01$ ; Fig. 4b). Finally, suppressor of cytokine signalling 4 (*SOCS4*) mRNA expression was also significantly increased between fetal and adult time-points ( $P < 0.05$ ; Fig. 4c). To assess the protein localisation of these signalling molecules, immunofluorescence was performed.

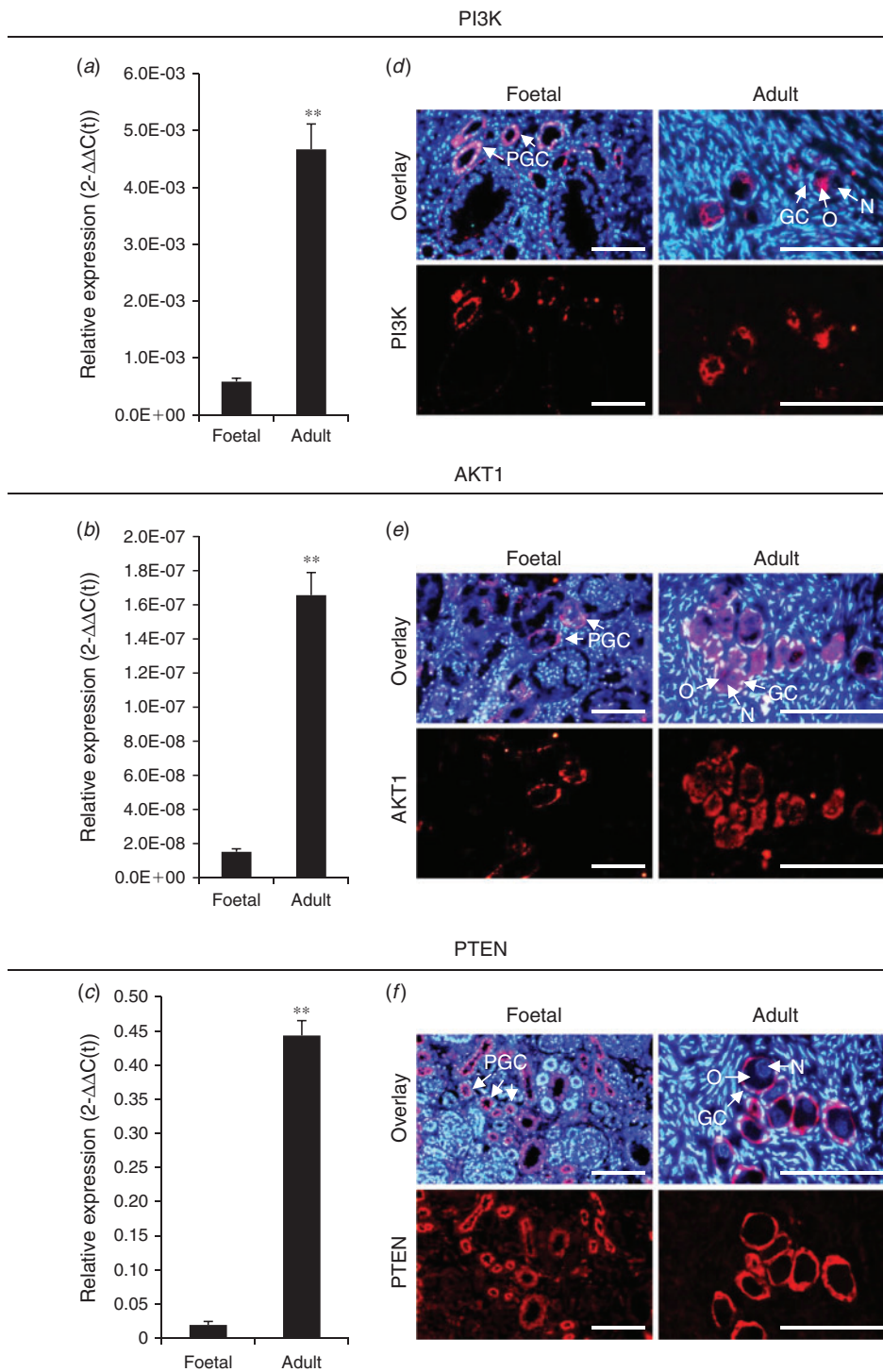
#### *JAK/STAT protein localisation changes during development in the mare ovary*

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. 4d). 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. 4e). A diffuse staining pattern for SOCS4 occurred in pregranulosa cells of the fetal ovary and persisted in granulosa cells in the adult ovary (Fig. 4f).

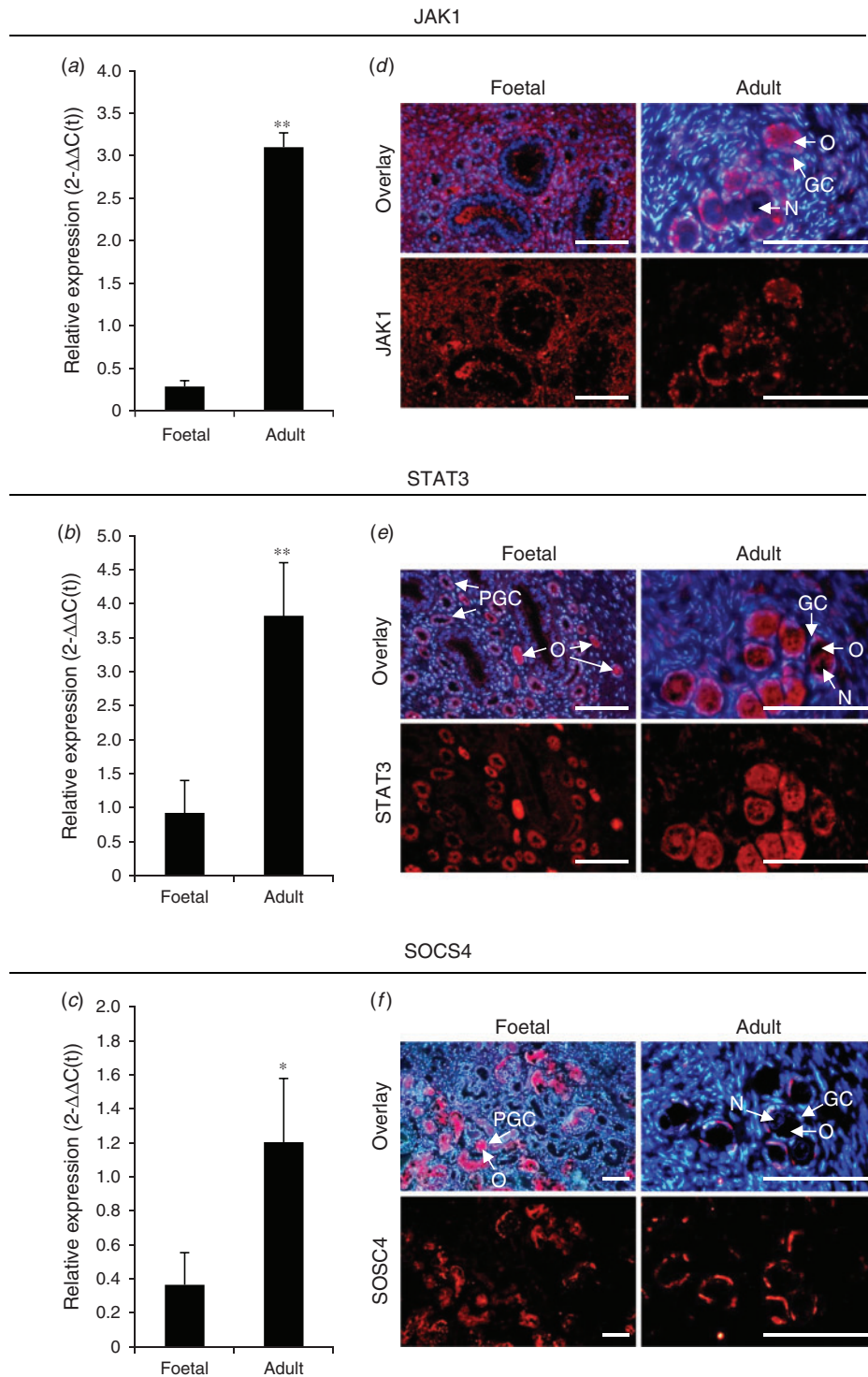
#### *Upstream and downstream signalling in the PI3K/AKT and JAK/STAT pathways*

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 foetal and adult ovaries compared with the reference gene, *GAPDH*. Data correspond to mean values  $\pm$  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 foetal 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 foetal 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  $\mu$ m.



**Fig. 4.** (a–c) Relative mRNA expression of (a) *JAK1*, (b) *STAT3* and (c) *SOCS4* between foetal and adult ovaries compared with the reference gene, *GAPDH*. Data correspond to mean values  $\pm$  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 foetal 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 foetal 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  $\mu$ M.

adult ovary (Fig. S1a–c). Upstream JAK/STAT factor CLCF1 labelled oogonia in fetal tissue and was expressed ubiquitously within the adult ovary (Fig. S1d). 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. S1e) and mTOR was expressed in pregranulosa cells in the fetal ovary and granulosa cells and oocyte cytoplasm in the adult ovary (Fig. S1f).

## 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.* 2006a).

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; Pastuszek *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.

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## **CHAPTER 3**

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

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## 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).



Research Article

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

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## 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<sub>2</sub>·2H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 mM NaHCO<sub>3</sub>, 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, Allerød, Denmark) and diluted to a final concentration of 20 × 10<sup>6</sup> 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.

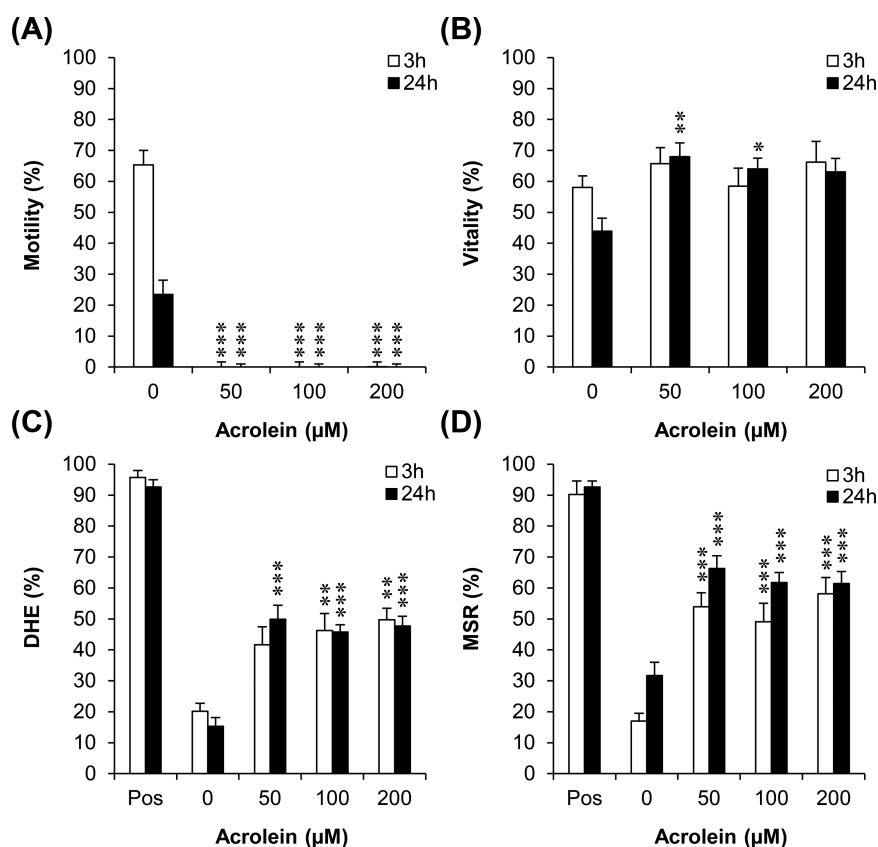
### Motility analysis

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 ≥ 50 μm/s and an STR of ≥ 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 long-pass (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  $\mu\text{M}$ ). Data correspond to mean values  $\pm$  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).

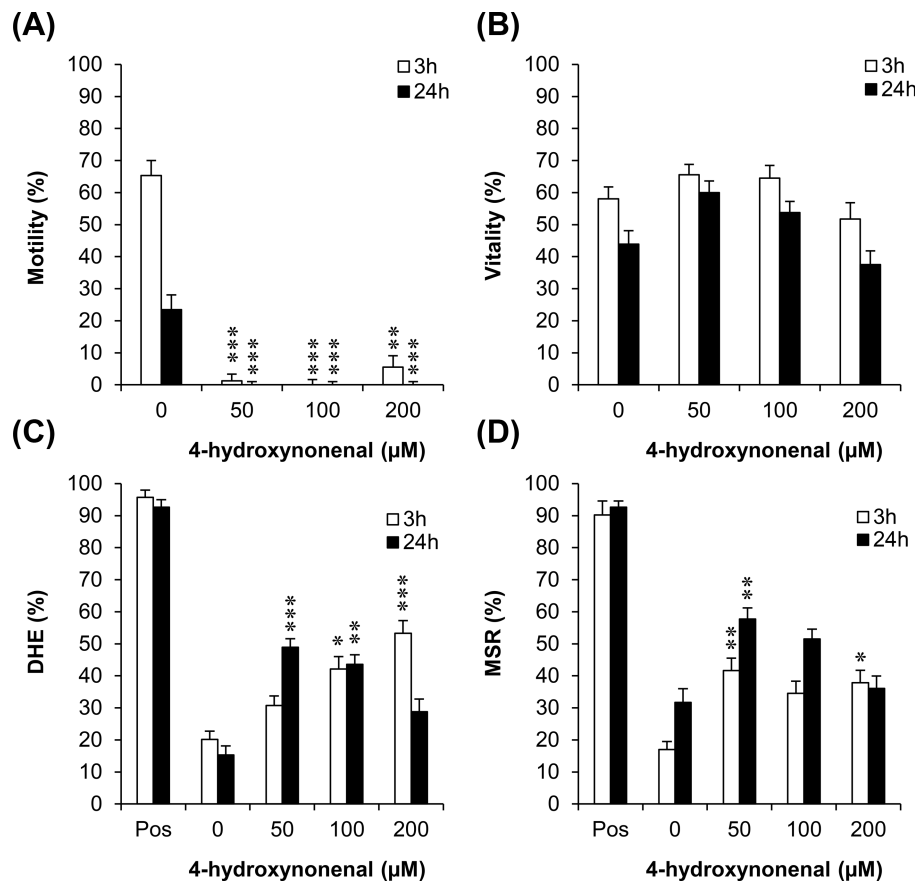
### Measurement of ALDH2 activity

ALDH2 activity was assessed in all 50  $\mu\text{M}$  aldehyde treatments using the Aldefluor assay kit (StemCell Technologies, Tullamarine, VIC). Live spermatozoa were centrifuged at 500  $\times g$  for 3 min and washed in BWW, then resuspended in Aldefluor buffer containing 5  $\mu\text{l/ml}$  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  $\mu\text{M}$  ALDI2 [44] for 10 min before being exposed to the respective 50  $\mu\text{M}$  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  $\times g$  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  $\times g$  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  $\times g$  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  $\times g$  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  $\times g$  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 ( $\sim 50 \times 10^6$  spermatozoa) following treatment with each aldehyde (50  $\mu\text{M}$ , 3 h incubation at 37°C) by resuspending pellets in 50  $\mu\text{l}$  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  $\times g$



**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  $\mu$ M). Data correspond to mean values  $\pm$  SEM; n = 9 independent ejaculates. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 for differences compared with untreated control sample.

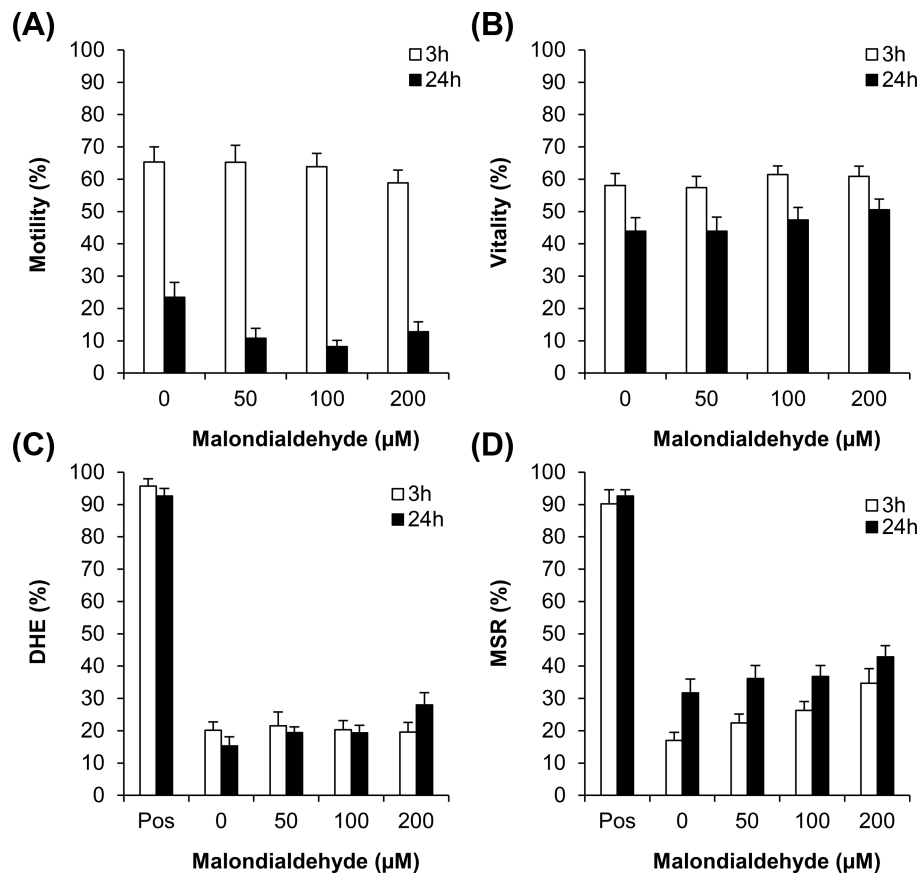
for 15 min at 4°C. The supernatant containing the soluble lysate was quantified using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) on a SPECTROstar nano (BMG Labtech, Ortenberg, Germany) and stored at -20°C for up to 1 month until required for western blotting. Protein extracted from spermatozoa was denatured with sodium dodecyl sulfate (SDS) loading buffer (0.2% SDS, 50% 0.375 M Tris, 10% sucrose, 4%  $\beta$ -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  $\mu$ M 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  $100 \times 10^6$  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  $14,000 \times g$  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  $CaCl_2 \cdot 2H_2O$ , 1.2 mM  $KH_2PO_4$ , 1.2 mM  $MgSO_4 \cdot 7H_2O$ , 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  $500 \times g$ ). 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  $10 \times 10^6$  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 BWB 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

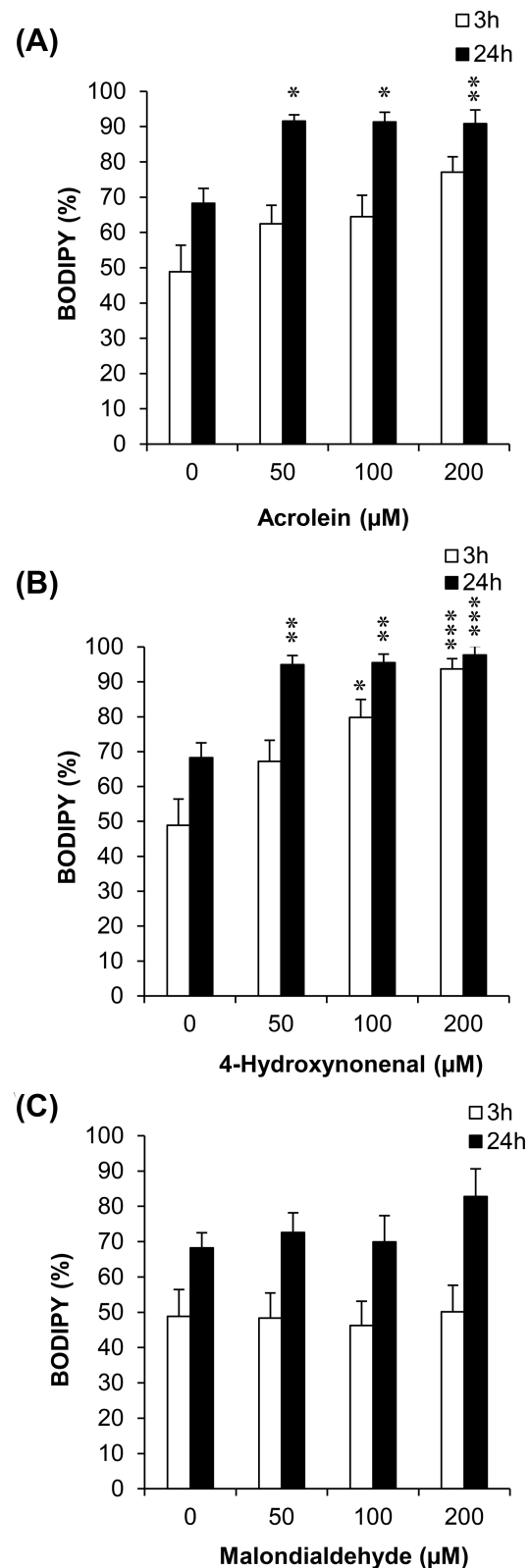
There was a significant effect of aldehyde treatment on sperm motility ( $P < 0.0001$ ), with no interaction between dose and time observed for ACR or 4HNE treatments. Stallion spermatozoa incubated with ACR suffered a rapid and complete loss of motility that was evident at the shortest time (3 h) and lowest dose (50  $\mu\text{M}$ ) 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.

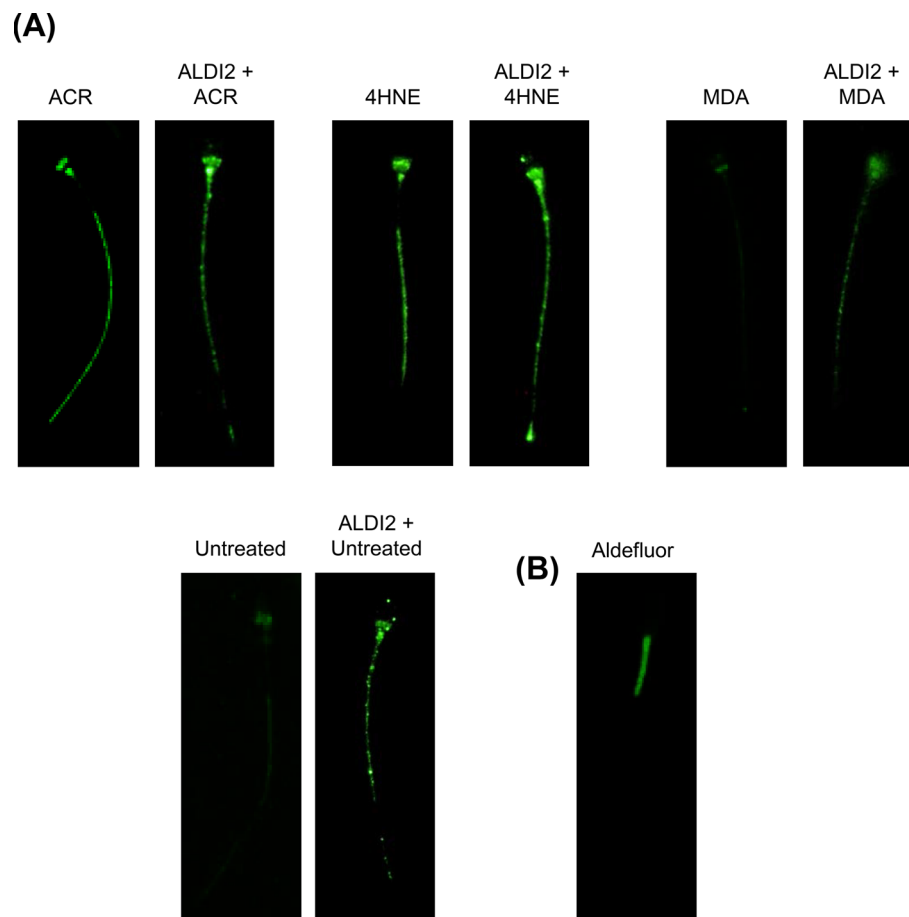
There was no impact on sperm motility following MDA exposure, compared with the control (Figure 3A). MDA failed to elicit any significant effect on sperm vitality (Figure 3B), total cellular ROS (Figure 3C), or mitochondrial ROS (Figure 3D), even after 24 h of exposure at the highest concentration.

### 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  $\pm$  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  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$ ; 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 pro-

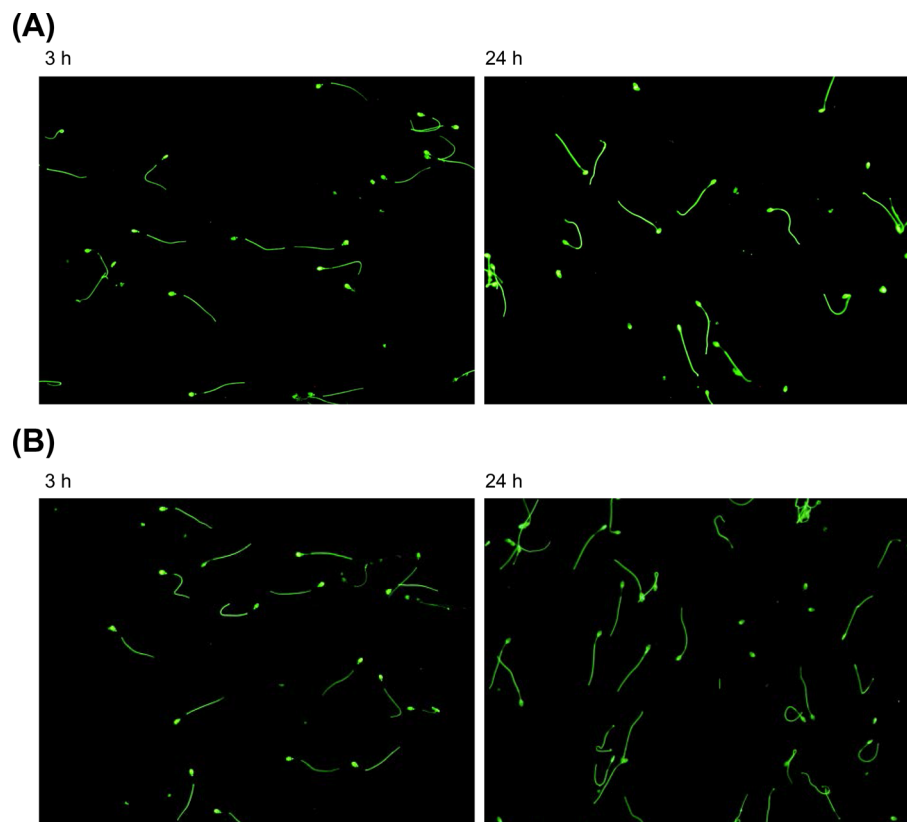
tection normally afforded to this critical sperm domain. In contrast to ACR and 4HNE, only relatively modest levels of MDA were able to be detected in stallion spermatozoa following their exposure to this aldehyde. In this regard, weak anti-MDA labeling was observed in the postacrosomal region; however, 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  $\mu\text{M}$  **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  $\sim 30$  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.

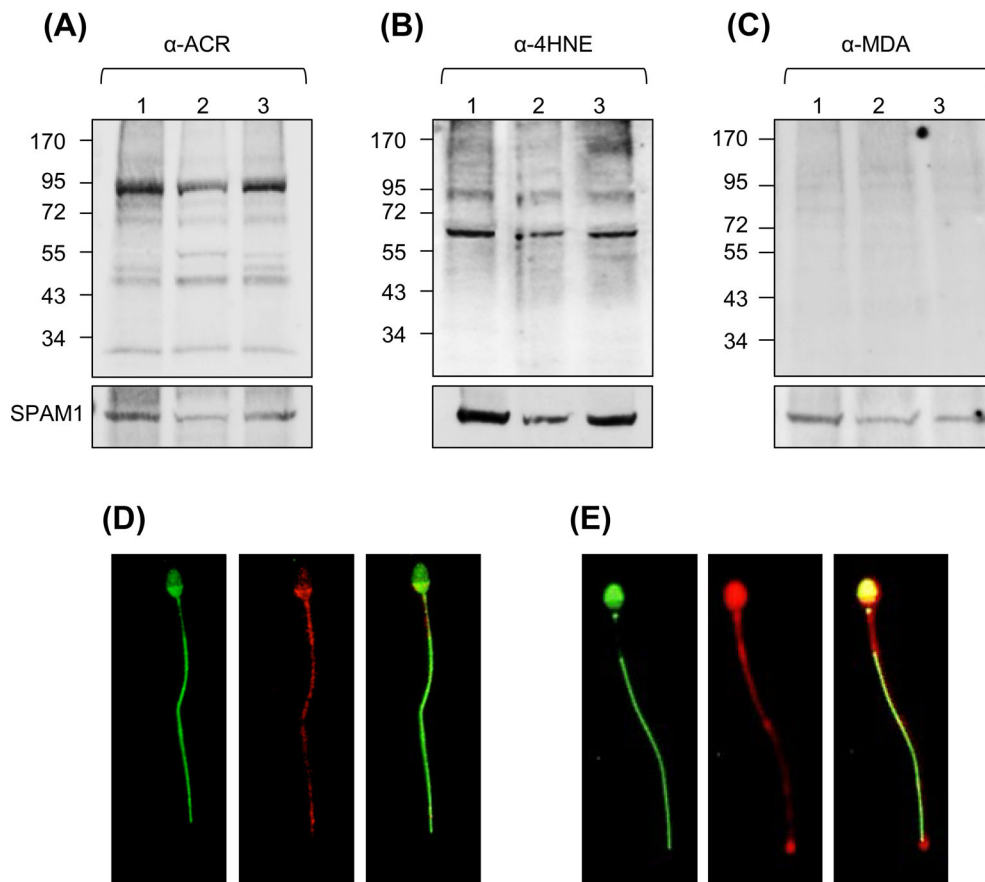
In the first approach, both HSP90AA1 and ARSA were colocalized with ACR and 4HNE, respectively. In fixed and permeabilized spermatozoa, HSP90AA1 was strongly labeled within the postacrosomal region of the head, midpiece, and principle piece of the sperm tail (Figure 7D). In comparison, ARSA was localized 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 ( $13.77 \pm 1.56\%$  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 ( $10.53 \pm 1.61\%$  and  $5.75 \pm 2.39\%$  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

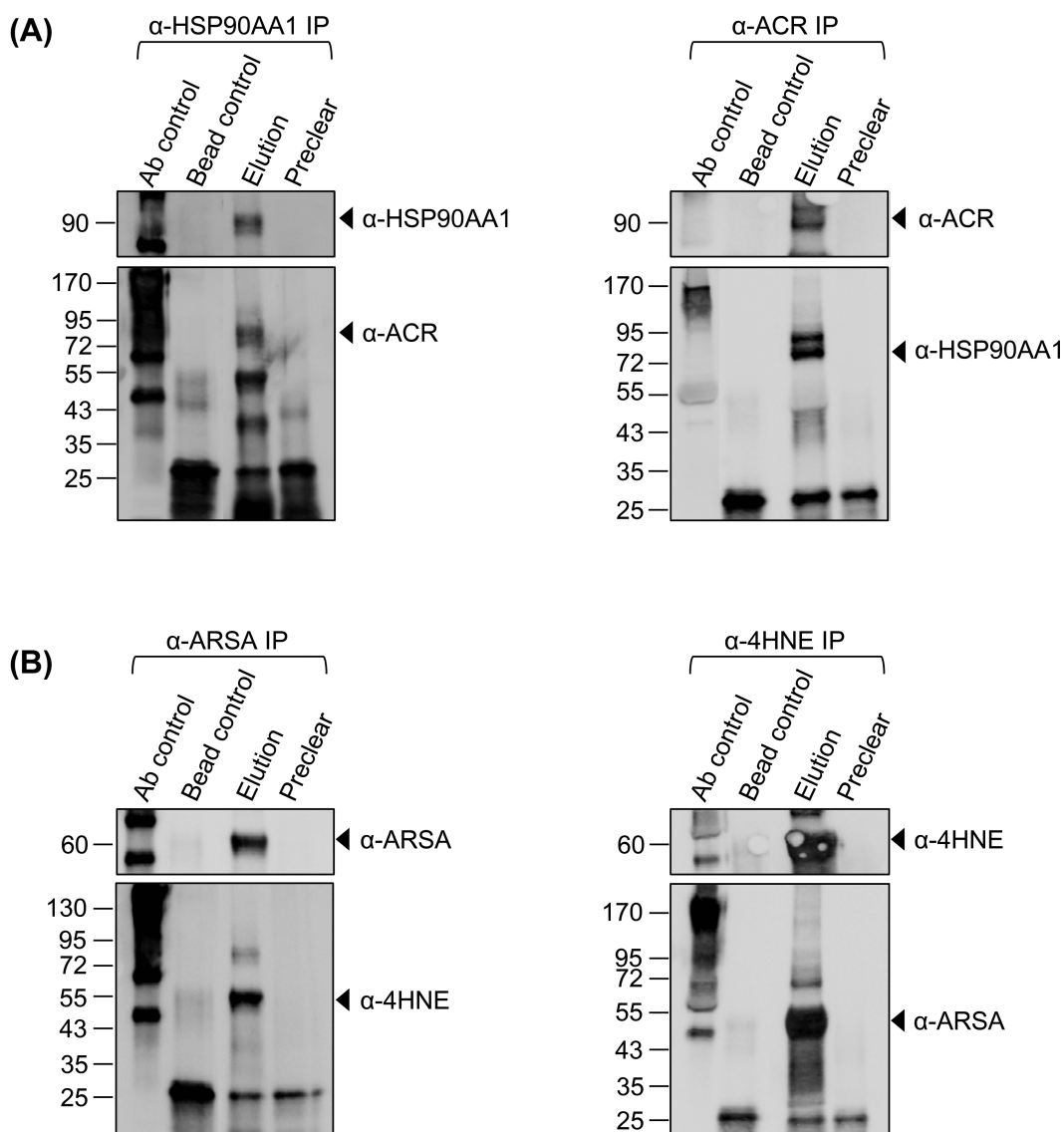


**Figure 7.** Western blot analysis of sperm protein lysates following exposure to 50  $\mu\text{M}$  **A)** ACR, **B)** 4HNE, and **C)** MDA at 37°C for 3 h. Each lane represents a different stallion. Loading control SPAM1. Fixed and permeabilized spermatozoa colocalized with **D)** ACR (Alexa Fluor 488; green) and HSP90AA1 (Alexa Fluor 594; red), and **E)** 4HNE (Alexa Fluor 488; green) and ARSA (Alexa Fluor 594; red) following 50  $\mu\text{M}$  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 mitochon-

drial 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).



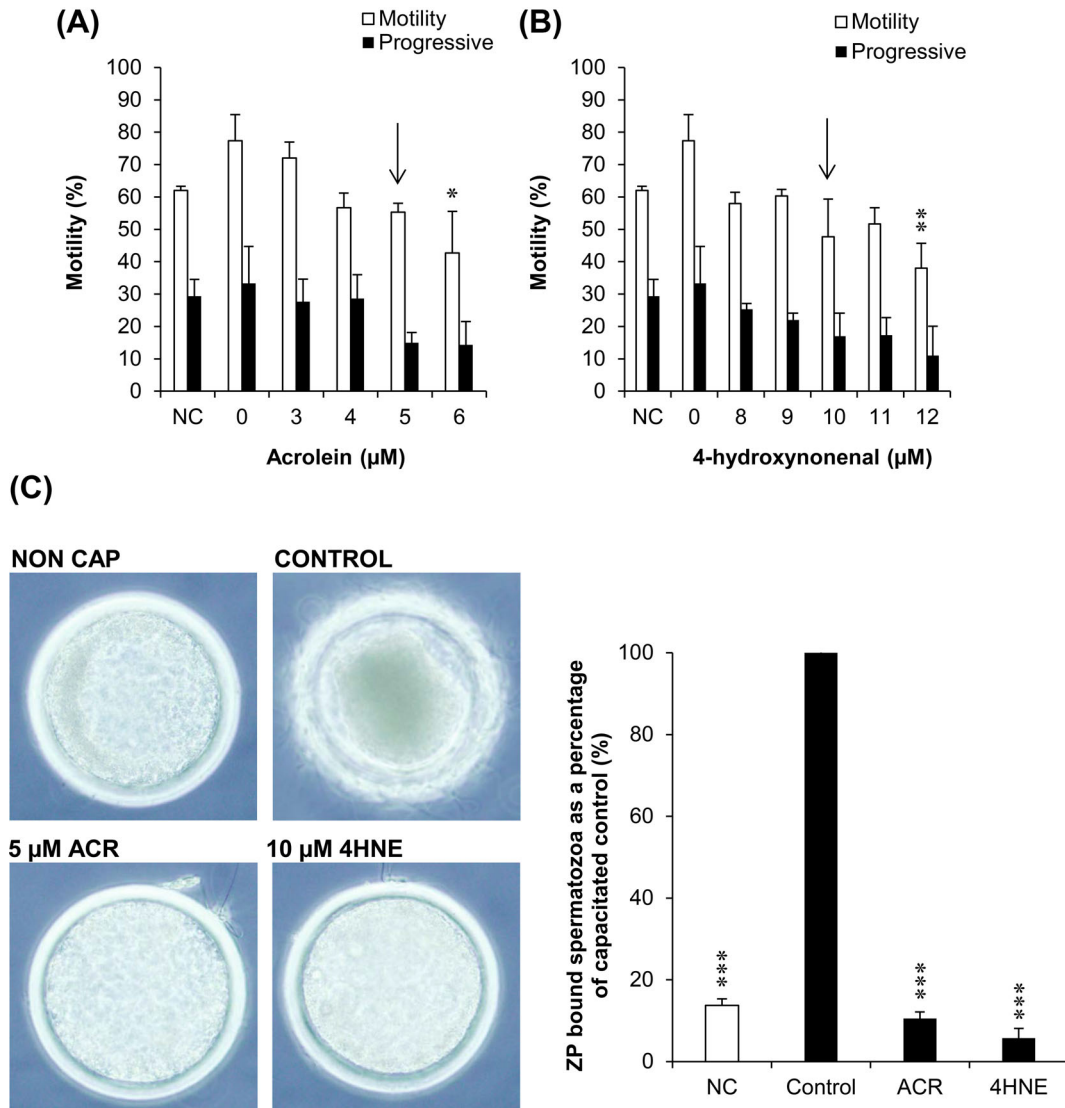
**Figure 8.** Reciprocal pull-downs and IP with **A)** HSP90AA1 and ACR, and **B)** ARSA and 4HNE following 50  $\mu$ M aldehyde exposure at 37°C for 3 h. Images are representative of all replicates ( $n = 3$ ).

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



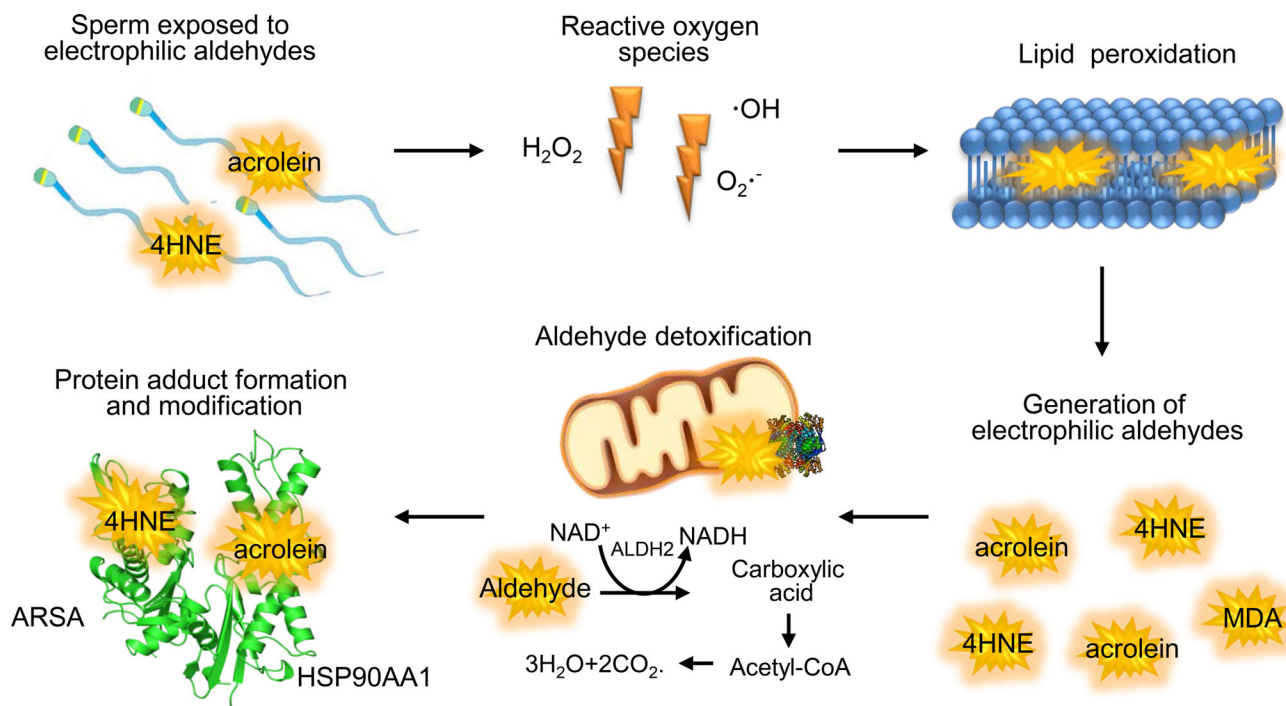
**Figure 9.** Total and progressive motility following a low dose-dependent exposure to **A)** ACR and **B)** 4HNE at 37°C for 3 h. **C)** Zona binding competence following exposure to 5 µM ACR and 10 µM 4HNE. Data correspond to mean values ± SEM; n = 3 independent ejaculates. \**P* < 0.05, \*\**P* < 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  $\mu$ M and 5 mM [38], whereas ACR production is estimated to occur between 30 and 180  $\mu$ M [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.

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## **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 fetuses 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<sup>†</sup>**

Running title: Immunization with covalently modified sperm proteins

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## **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:  $4.66 \pm 4.66$  vs control:  $9.33 \pm 4.70$ ). The antibodies elicited bound predominantly to the periacrosomal region of the sperm head and significantly decreased sperm-zona pellucida binding competence *in vitro* (acrolein:  $15.48 \pm 0.96\%$  vs control:  $100 \pm 5.59\%$ ,  $P < 0.0001$ ). 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  $\mu$ l (400  $\mu$ g/ml) non-adducted, acrolein-adducted or 4HNE-adducted sperm lysate or 25  $\mu$ l 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  $\times$  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°C for 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<sub>2</sub> 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<sub>2</sub>·2H<sub>2</sub>O, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 mM NaHCO<sub>3</sub>, 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  $10 \times 10^6$  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 diazobicyclo-[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  $\geq 10 \mu\text{/s}$  were considered progressive. A minimum of 200 spermatozoa in five fields were assessed using 80  $\mu\text{m}$  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  $\times g$  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  $\times g$  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  $500 \times g$  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  $500 \times g$  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  $37^{\circ}\text{C}$ . Oocytes were separated from adherent cumulus cells by gentle pipetting and washed three times in BWW. Oocytes were stored at  $4^{\circ}\text{C}$  in a high salt storage medium consisting of 1.5 mM  $\text{MgCl}_2$ , 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  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  : 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 dibutyl cyclic adenosine monophosphate. Cells were incubated at  $37^{\circ}\text{C}$  under an atmosphere of 5%  $\text{CO}_2$  : 95% air. Non-capacitated cells were incubated for the same period of time in BWW prepared without  $\text{NaHCO}_3$ . Spermatozoa were diluted to a concentration of  $1 \times 10^6$  cells/ml and 20  $\mu\text{l}$  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) polyvinylpyrrolidone 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<sub>2</sub>O:CH<sub>3</sub>CN (98:2; + 0.1% formic acid) to H<sub>2</sub>O:CH<sub>3</sub>CN (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  $100 \times 10^6$  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  $14\,000 \times g$  at 4°C for 20 min, and added to 50  $\mu$ l aliquots of washed protein G Dynabeads to preclear at 4°C for 1 h under constant rotation. Meanwhile, 10  $\mu$ g 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*<sup>-/-</sup>) 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.

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## FIGURE LEGENDS

**Figure 1.** *In vivo* experimental design. Mouse spermatozoa were left untreated, or treated with 50  $\mu$ M acrolein or 50  $\mu$ M 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  $\mu\text{M}$  acrolein or 50  $\mu\text{M}$  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  $\pm$  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  $\pm$  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  $\mu$ M 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).

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

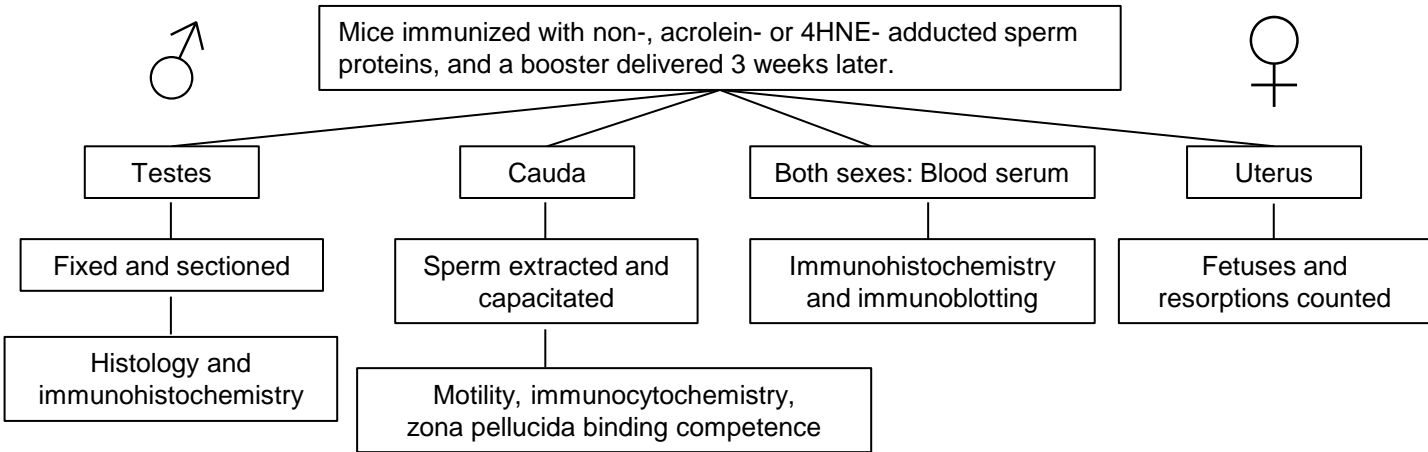
TABLE 2. Identification of potential targets for acrolein adduction <i>in vitro</i>				
Protein (symbol)	Mascot score	Peptides matches	Peptide sequences	UniProt accession number
Glucose-6-phosphate isomerase	944	37	11	P06745
Protein disulfide-isomerase A3 (PDIA3)	685	19	12	P27773
Protein disulfide-isomerase 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 <i>in vivo</i>				
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



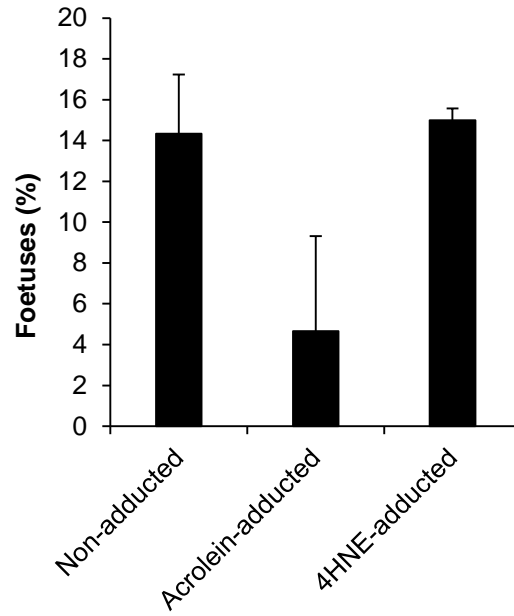
Supplementary table 1. Identification of potential targets for 4HNE adduction <i>in vitro</i>				
Protein (symbol)	Mascot score	Peptides matches	Peptide sequences	UniProt accession number
Aspartate aminotransferase (AATM)	528	21	10	P05202
Phosphoglycerate kinase 2 (PGK2)	467	17	9	P09041
Solute carrier 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

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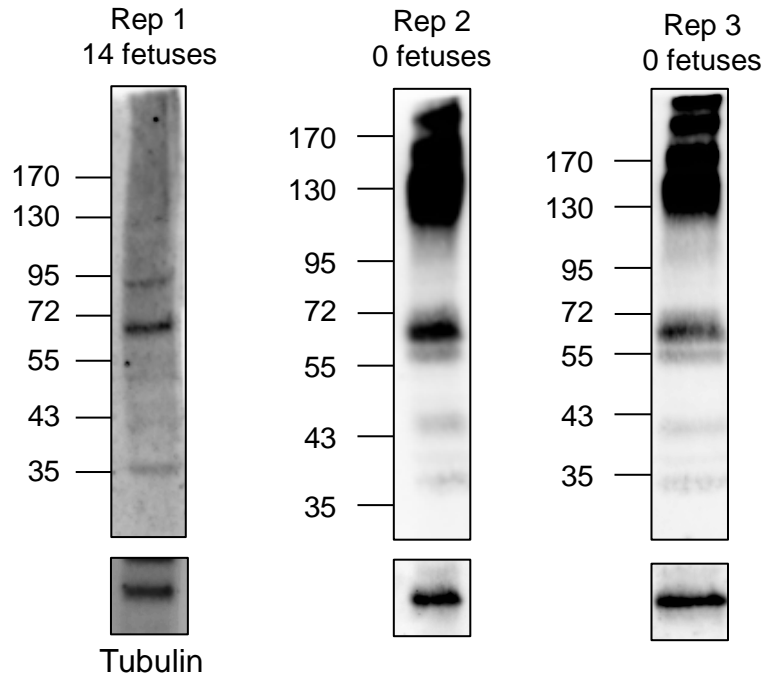


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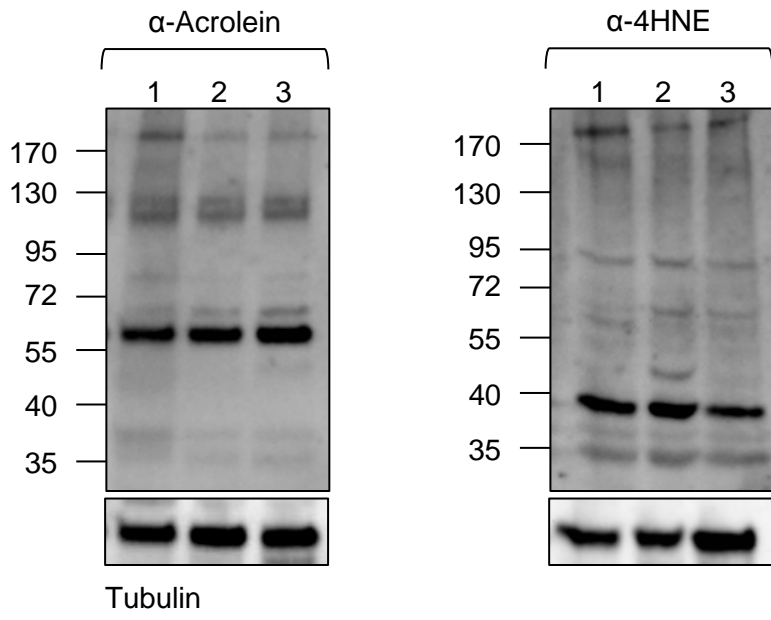
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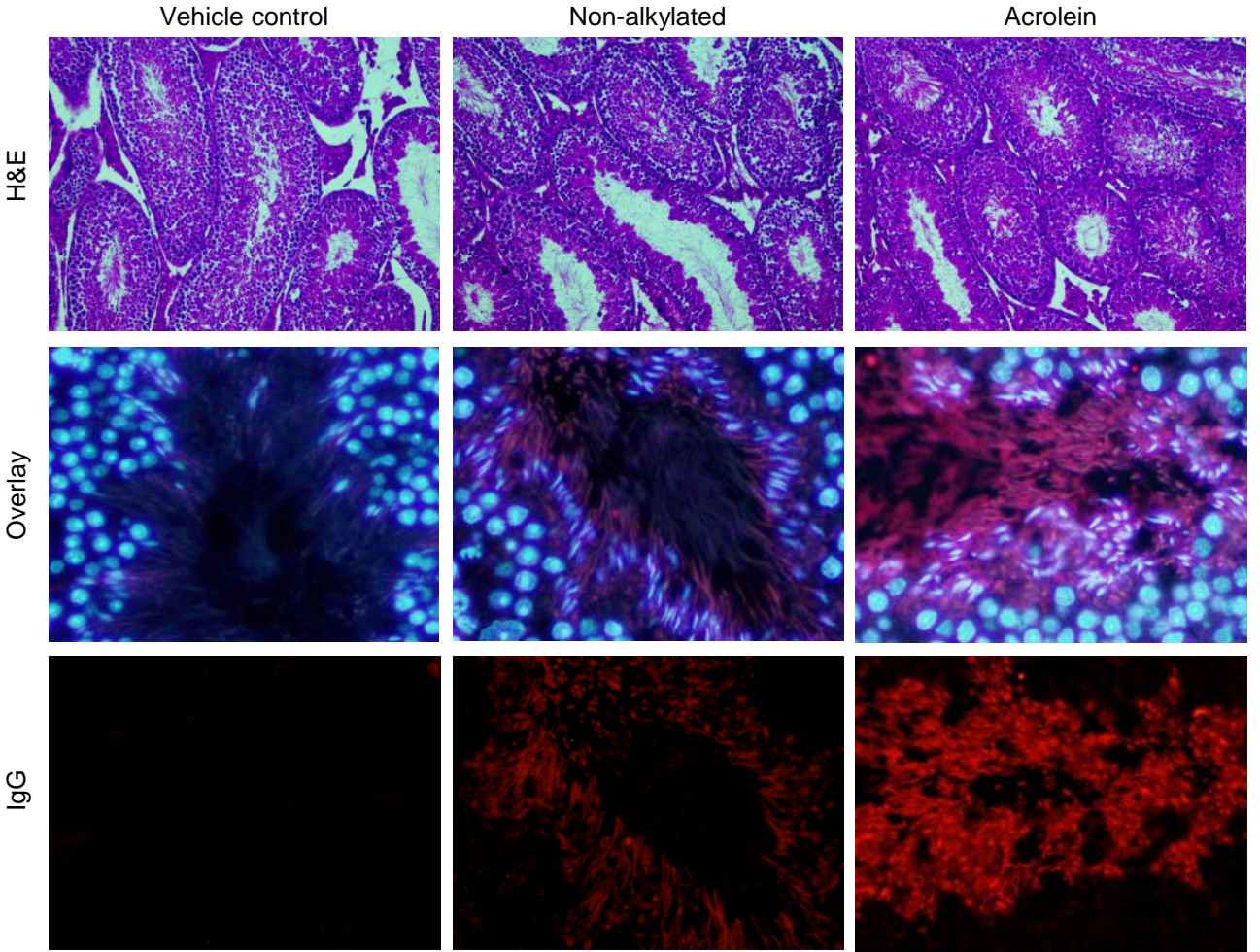
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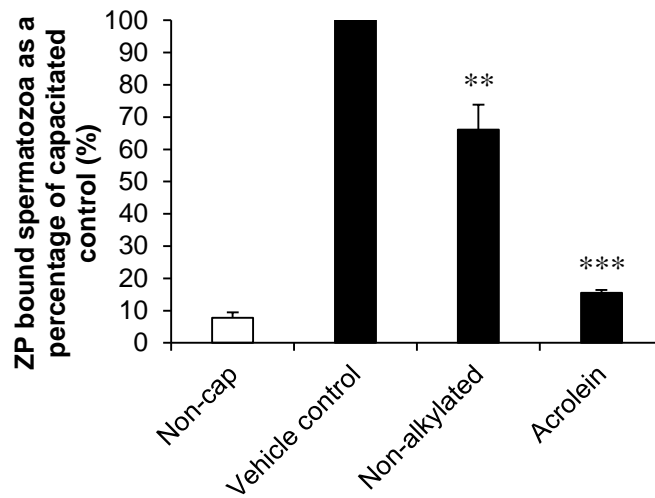
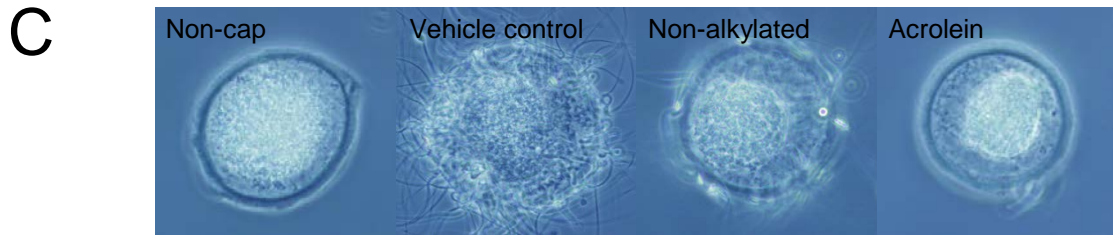
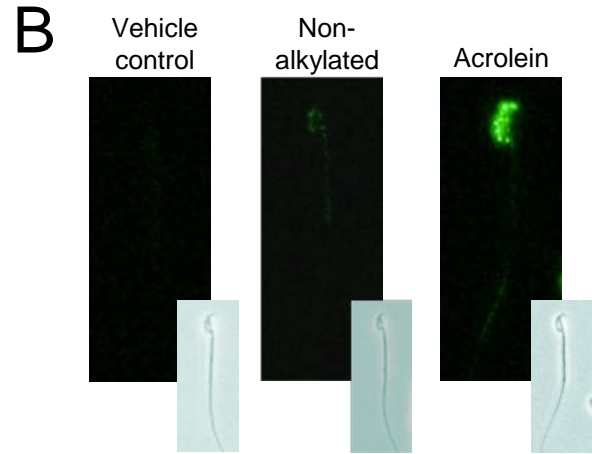
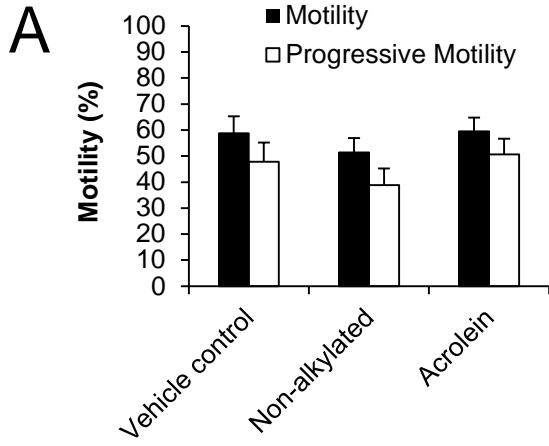
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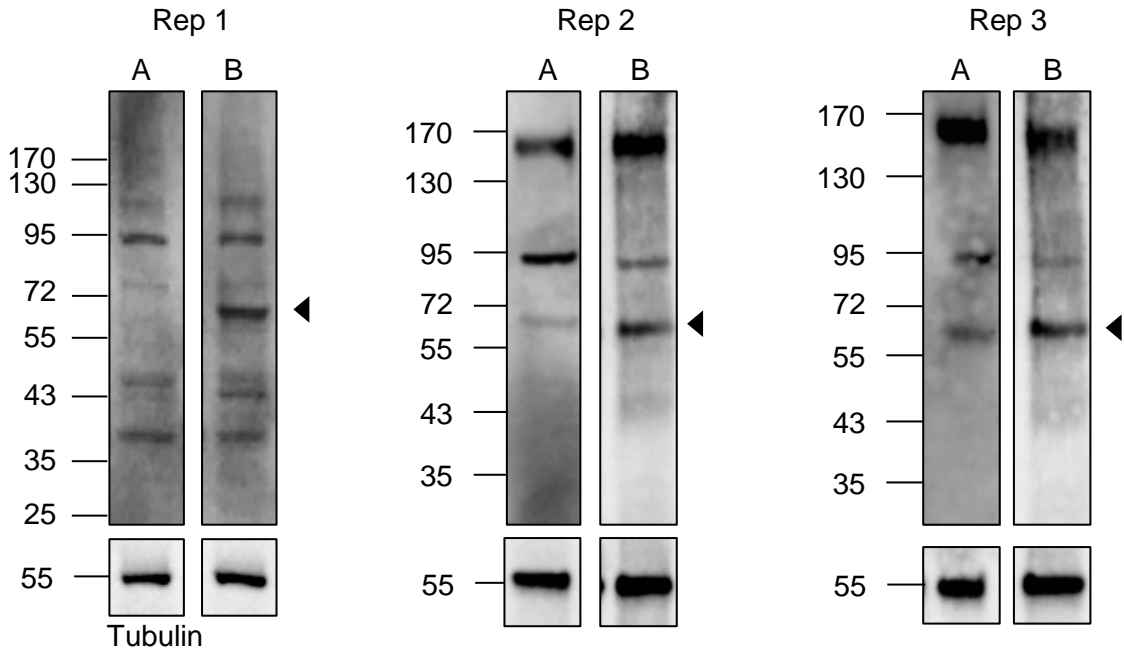
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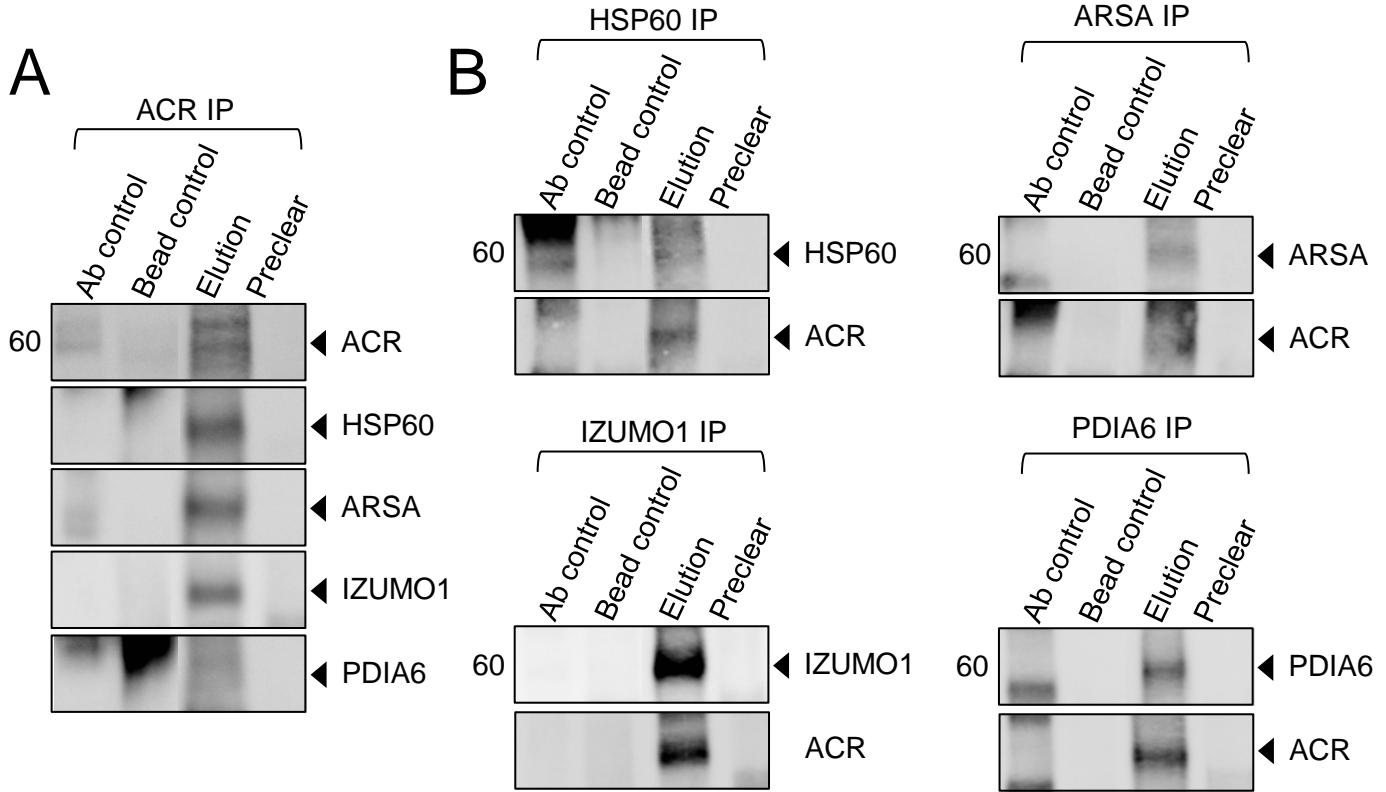
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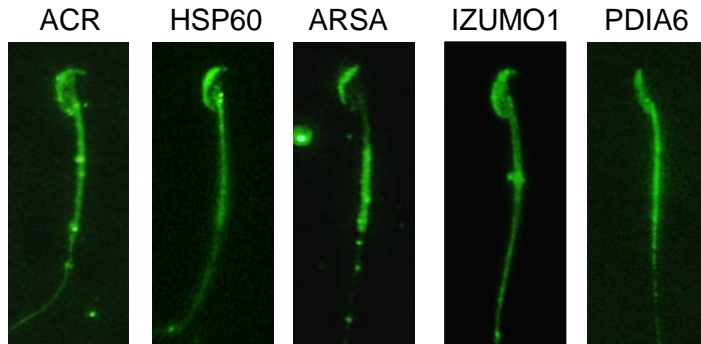
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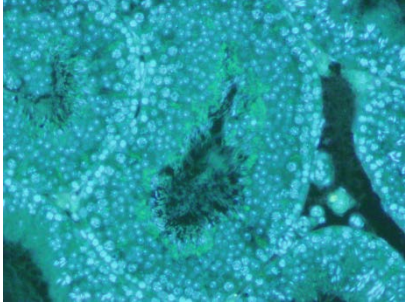
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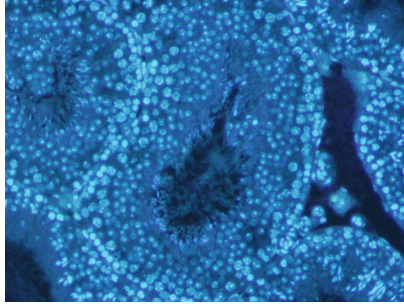


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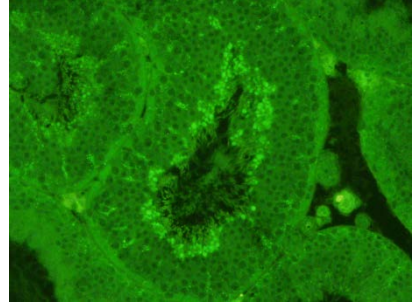
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## **CHAPTER 5**

## **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 in 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, PDIA5). 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.

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