Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/evj.12512

Editorials

Science-in-brief: Report of the Havemeyer Foundation W.R. (Twink) Allen Symposium on Equine Fertility and Assisted Reproduction

Introduction

In mid-June 2015, the Dorothy Russell Havemeyer Foundation convened a 2 day Symposium on Equine Fertility and Assisted Reproduction in Krakow, Poland to celebrate the 50 year scientific career of Emeritus Professor W.R. (Twink) Allen and his longstanding and fruitful collaboration with Emeritus Professor Marian Tischner and his equine reproduction team in Krakow. The meeting, chaired by Professor Tischner and Dr Piotr Micek of the University of Agriculture in Krakow, comprised 6 themed sessions and was attended by 37 scientists from 17 countries. The veterinary and scientific highlights included:

Twink Allen's career

Dr Peter Rossdale (Newmarket, UK), who was unable to attend the Symposium personally, submitted an amusing written account of Twink's scientific career in Cambridge and Newmarket, highlighting his contributions to equine reproductive physiology and medicine, especially as they benefitted the daily work of the Thoroughbred studfarm veterinarian. Dr Bob Moor FRS (Cambridge, UK), Professor Doug Antczak (Cornell University, USA), Dr Sandra Wilsher (Newmarket, UK), Professor Marian Tischner (Krakow, Poland) and Richard Greenwood (Newmarket, UK) all reviewed aspects of Allen's contributions to equine and wild animal reproductive physiology. These included:

- Discovery of the specialised, binucleated, trophoblast cells of the chorionic girdle portion of the equine fetal membranes as the source of equine chorionic gonadotrophin (eCG) and their invasion of the maternal endometrium at Days 36–38 of gestation to form unique equine endometrial cups [1].
- Definition of the relative roles of paternal genotype and uterine environment upon the development and eCG secretion rates of the endometrial cups in intra-, inter- and extraspecies equine pregnancies.
- Factors influencing maternal, humoral and cell-mediated immune recognition of, and responses to, foreign antigens expressed by the fetal endometrial cup cells and normal noninvasive trophoblast of the allantochorion [1].
- The roles of eCG and pituitary gonadotrophic hormones in stimulating secondary follicular and luteal development in the mare's ovaries during the first half of pregnancy.
- The role of the fetal gonads in the secretion of oestrogens by the placenta during the second half of equine pregnancy.
- Development of the techniques of embryo recovery, transfer, *in vivo* transport, freezing, bisection, between species transfer, *in vitro* culture, intracytoplasmic sperm injection (ICSI) and nuclear transfer [2].
- Factors influencing implantation, placental development and fetal growth in the mare [3].
- Factors influencing spermatogenesis and the collection and deep freezing of stallion semen.
- The development and application of hormonal therapies and diagnostic techniques to Thoroughbred breeding, including prostaglandins, progestagen withdrawal, gonadotrophin-releasing hormone (GnRH) analogues, ultrasound scanning of the ovaries and uterus to monitor follicular growth, ovulation and pregnancy, uterine videoendoscopy, intrauterine Nd:YAG laser therapy and rigid laparoscopy for unblocking mare oviducts.
- Studies on placentation and maternal and fetal gonadal functions in support of pregnancy in the African elephant [4], giraffe, zebra, wildebeest and hedgehog.

Physiology of equine reproduction

Dr Janet Roser (Davis, USA), Professor Barry Ball (Lexington, USA), Dr Anne-Cecile LeFranc (Lyon, France), Professor Tom Stout (Utrecht, The Netherlands), Dr Julia Kydd (Nottingham, UK), Professor Kazuyoshi Taya (Tokyo, Japan), Dr Domingo Tortonese (Bristol, UK), Professor Abigail Fowden (Cambridge, UK), Dr Mandi de Mestre (London, UK), Dr Myriam Boeta (Mexico City, Mexico) and Dr A Martinez (Mexico City, Mexico) all contributed to this session and reported that:

- Recombinant-derived equine follicle stimulating hormone (reFSH) and luteinising hormone (reLH) have both now been synthesised and trialled in mares. Treatment of cycling mares with reFSH followed by reLH results in 4.6 ± 4.9 ovulations which yield 3.9 ± 0.9 embryos when the mare is flushed on Day 8, and treatment of seasonally anoestrous mares with reFSH results in a fertile ovulation within 6 days.
- Anti-Müllerian hormone (AMH) is a granulosa-cell derived glycoprotein, the assay of which in mare's blood correlates well with the number of 6–20 mm diameter antral follicles in her ovaries, although the relationship is influenced by mare age. The concentration of AMH in the fluid of dominant, but not growing, follicles declines as the concentration of oestradiol increases.
- The density of endometrial glands in the mare's uterus increases during dioestrus and especially during the first 30 days of pregnancy. Furthermore, the size and number of secretory vacuoles in the cytoplasm of lumenal and glandular epithelial cells is appreciably higher at 30 days of gestation than at the equivalent stage of prolonged dioestrus.
- Embryonic development is retarded following transfer to negatively asynchronous recipient mares. Trophectodermal mRNA expression of glucose and amino acid transporter genes and of paternally imprinted genes is reduced in the yolk sac of such asynchronously transferred embryos.
- Equine herpesvirus-I (EHV-I) becomes intracellular within a few hours of contact with the respiratory mucosa of an infected horse and reaches the bronchial lymph nodes within 12 h. Protective immune responses include both humoral and cell-mediated components and EHV-I specific cytotoxic T lymphocytes can lyse virus-infected cells.
- Prolactin concentrations in stallion blood are highest during the breeding season. They are much lower in geldings but still show the same seasonal pattern. The increase in prolactin levels in youngstock is also remarkably seasonal, indicating that the HPA is already responsive to photoperiod. In Spring-mated mares, peak serum prolactin levels occur around the 10th week of pregnancy whereas they are delayed to 20–40 weeks gestation in Autumn-mated mares. Therefore, prolactin release follows photoperiod rather than stage of pregnancy. In a similar manner, yearlings maintained during the winter under a 14.5L:9.5D artificial light regime shed their coats sooner and showed a higher serum gonadotrophin and steroid hormone concentrations than nonlit counterparts. All these changes appear to be stimulated by elevated prolactin which is clearly photoperiod-dependent.
- The mechanisms for microvascular remodelling in the mare's pituitary gland are highly seasonal and these mechanisms are regulated by gonadal feedback.
- The main rise in glucocorticoids in the horse foal occurs after, not before, birth indicating that the horse is more susceptible to glucocorticoid programming as a neonate. Post natal glucose homeostasis and hypothalamic-pituitary-adrenal (HPA) functions can be influenced by early life glucocorticoid overexposure. Therefore, neonatal glucocorticoids are very important in programming metabolic and endocrine phenotype in young horses.

- The chorionic girdle undergoes 3 phases of development; 1) rapid trophoblast proliferation, 2) differentiation of uninucleate into binucleate trophoblast cells expressing eCG and 3) an invasive phenotype to penetrate the endometrium. Glial cells missing 1 (GCM1) regulates eCGβ expression in the girdle, as do a number of the 127 transcription factors induced during girdle development.
- Pregnant mares treated weekly with a GnRH antagonist between 35 and 80 days of gestation developed a higher proportion of unruptured luteinised follicles than normal secondary ovulations in their ovaries, but the normal balance was restored if they were also treated with hCG (Chorulon) when accessory follicles reached >35 mm diameter. The conclusion is therefore drawn that a functional pituitary gland is necessary for secondary ovulations to occur in early pregnant mares.
- In 4 Jenny donkeys inseminated with horse semen so that they carried hinny conceptuses, several secondary and accessory corpora lutea started to appear in the maternal ovaries by Day 56 of gestation and the mean number had risen to as high as 18.5 by Day 120. These multiple luteal structures gave rise to maternal serum progesterone concentrations as high as 1940 ng/ml by Day 84.

Embryo transfer and embryo technologies

Dr Robert Pashen (Melbourne, Australia), Dr Santos Suviria (Argentina) and Dr Julio Oriol (Abu Dhabi, UAE) presented aspects of embryo transfer (ET) and related technologies in the mare. Dr Eric Palmer (Paris, France) submitted an additional abstract on cloning in European Sport horses and, in this session, the following topics were discussed:

- Originally, in the early 1970s, horse embryos were recovered surgically from, and transferred surgically to, the oviduct to give recovery and pregnancy rates of >85%. Early nonsurgical recovery rates of ±30% from flushing only the ipsilateral uterine horn doubled when the whole uterus was flushed. Nowadays, advances in reproductive technologies such as embryo freezing and vitrification, ICSI, AI with sex-sorted semen and even cloning are being employed in South American sport horses, especially polo ponies, of which >10,000 have been born following embryo transfer over the past 40 years.
- The widespread use of ET in polo ponies over the last 20 years has resulted in a higher supply of youngstock of more consistent quality. Trainers have noted improved docility, tranquillity and trainability in ET youngstock and, for the top 4 teams in the world competing in the last 3 Argentine Open Championships, 71% of the ponies played have resulted from ET.
- Attempted embryo recovery from aged Arabian mares in the Middle East resulted in a 65% recovery rate from those aged younger than 15 years, 42% from mares aged 15–19 years and only 23% from mares aged greater than 19 years. Overall, 60% of normal, nonlaminitic mares produced an embryo when flushed whereas only 18% of mares exhibiting chronic laminitis produced an embryo, regardless of age.
- Following nuclear transfer (cloning) in European Sport horses, 75% of cloned pregnancies are aborted between first pregnancy diagnosis and term. Denmark and the UK are the European countries most negative towards the application of cloning whereas both North and South America readily accept this novel technique. Three Sport horse studbooks in Europe now register cloned foals and, in 2012, the Federation Equestrian Internationale (FEI) approved the entry of cloned horses in FEI-sponsored competitions.

Stud medicine

Dr Walter Zent (Lexington, USA), Dr Lee Morris (Te Awamutu, NZ) and Professor Terttu Katila (Helsinki, Finland) shared clinical data and studies showing:

- On one intensively managed studfarm housing 70 mares per year over 8 years, employing all the reproductive techniques and veterinary interventions developed over the past 50 years and paying particular attention to management factors such as dates of foaling and first covering, matings per conception and percentage pregnant and live foal birth rates, led to improved reproductive performance for that herd that made breeding more efficient and profitable than in comparable farms that managed mares less intensively.
- Analysis of a subset of 84 Thoroughbred mares standing at one of 6 studfarms in the North Island of New Zealand indicated that ambient temperature, light intensity, atmospheric pressure and rainfall had no significant influence on the growth rate of ovarian follicles whereas the date when follicle growth commenced did exert an effect. Human chorionic gonadotrophin (hCG; Chorulon) and the GnRH analogue, deslorelin, were equally effective in stimulating ovulation of the dominant follicle between 24 and 48 h after administration.
- Repeated treatment of dioestrous mares with oxytocin prevents luteolysis, suggesting that oxytocin receptors have a role in luteostasis and maternal recognition of pregnancy (MRP) in the mare. Both the endometrium and conceptus produce PGF2α in pregnant mares and the latter secretes PGE2 until at least Day 32. Experimental evidence indicates that prostaglandins (PGF2α or PGE2), oxytocin and embryonic oestrogens are not involved in MRP whereas all 4 components are likely vital for embryo motility between Days 6 and 16 after ovulation and for improving embryonic uptake of nutrient-rich endometrial histotroph. The identity of the MRP signal in the mare remains an intriguing mystery.

The stallion and pregnancy loss

Martin Boyle (Cambridge, UK), Dr Mats Troedsson (Doha, Qatar), Professor Domenik Burger (Avenches, Switzerland), John Newcombe (Walsall, UK) and Dr Judy Cawdell-Smith (Gatton, Australia) discussed their work in this session. Professor Ed Squires (Lexington, USA) submitted an abstract but was unable to attend the Symposium in person.

- Repeated collections of semen from 122 stallions of various breeds and types 2–4 times per week during Autumn showed, surprisingly, that the total number of spermatozoa per ejaculate declined with time in only 49% of the horses while it remained constant in 38% and actually rose in 13%. Native Pony breeds were the most surprising in that spermatozoa per ejaculate remained constant in 38% and showed a rise in 44%; in only 3 ponies did sperm output actually fall towards the winter.
- Multi-site analysis of fertility rates in mares inseminated with frozen-thawed stallion semen indicated a 64% pregnancy rate in those inseminated with 9 or more straws of semen, 48% with 5–8 straws and 44% with 1–4 straws. Mares inseminated twice during oestrus were 1.65 times more likely to conceive than those inseminated once; insemination before and after ovulation gave a 55% pregnancy rate vs. 39% for once before and 47% for once after, ovulation. Insemination into the body of the uterus gave a 53% pregnancy rate compared with only 44% in mares that underwent ipsilateral deep uterine horn insemination. Thus, mares inseminated with 800 million spermatozoa into the body of the uterus before and after ovulation have the highest chance of conceiving.
- Spermatozoa are eliminated from the mare's uterus by a combination of myometrial contractions and phagocytosis by polymorphonuclear leucocytes (PMNs). Experiments have demonstrated that the seminal plasma proteins, CRISP-3 and lactoferin, are involved in, respectively, selecting live spermatozoa for transport to the mare's oviducts while at the same time promoting the elimination of dead spermatozoa from the uterus.
- Behaviour trials indicate that free-ranging stallions perform 'strategic ejaculation' by restricting their matings to higher quality mares and those that differ most from themselves at the major histocompatibility complex (MHC) gene loci. Typically, both mares and stallions will avoid MHC-similar matings so as to increase the overall heterozygosity of their offspring.
- While many aspects of studfarm veterinary management have resulted in striking advances in fertility over the past 50 years, early embryonic loss (EEL) rates have remained unchanged. The rate of EEL varies between 6 and 9% in younger mares but can rise to as high as ±50% in old mares, presumably due to fertilization of aged oocytes leading to lethal karyotypic abnormalities. Early embryonic loss is higher in mares served on the foal heat and in those in which the conceptus lodges in the previously gravid uterine horn. Trials have shown that EEL is not caused by low maternal serum progesterone concentrations, nor by the presence of age-related endometrial degeneration (endometrosis). The

most useful veterinary therapy to combat EEL is frequent ultrasound scanning during the first 40 days after insemination to diagnose its occurrence early and thereby be able to treat the affected mare with PGF2α and get her re-mated quickly.

- Focal mucoid placentitis (FMP) occurs commonly in Thoroughbred mares in Kentucky, USA where it is usually associated with Nocardioform bacteria. Pregnant mares in Queensland, Australia dosed orally with 5 g of the exoskeletons of processionary caterpillars daily for 5 days between either 25–30 or 46–51 days of gestation exhibited typical caterpillar-induced abortions between 5 and 15 days after the start of treatment in 3 mares. Two other mares exhibited typical FMP lesions unassociated with the cervical star at or near term from which Rhodococcus Sphingomonas and Streptophomonas spp. bacteria were isolated. This suggests strongly that focal placentitis lesions in mares originate from soil or gut bacteria transported to the placental interface by caterpillar setae migrating through the apposing walls of the large colon and gravid uterus.

Reproduction in other species

The Symposium concluded with 5 fascinating papers on aspects of health and reproduction in species other than the horse. Speakers included Dr Su Metcalf (Cambridge, UK), Professor Xihe Li (Huhot, Inner Mongolia), Dr John Zhans (New York, USA) Dr Lulu Skidmore (Dubai, UAE) and Dr Imke Lueders (Hamburg, Germany).

- Multiple sclerosis (MS) is an incurable autoimmune disease of the central nervous system in humans. Leukaemia inhibitory factor (LIF) is a stem cell growth promoter that stimulates myelin repair and suppresses autoimmune attack. Leukaemia inhibitory factor nano (LIFnano) uses biodegradable, biocompatible nanoparticles made from B2GA, the material used to make soluble sutures. LIFnano delivers LIF to specific sites in the body. It is efficacious as a neuroimmune modulator *in vivo*.
- The ability to sort spermatozoa into X chromosome(female) and Y chromosome(male)-bearing populations has influenced the dairy industry worldwide. Modern sorting machines can sort spermatozoa at a rate of 5000–7000/s with >90% accuracy. This means that each sorter is limited to producing 30–50,000 insemination doses of sorted spermatozoa per year. However, mixing sorted spermatozoa with xenosemen (from another animal species) to reduce number of sexed but maintain total number of sperm per dose almost doubles the production rate to 60–80,000 doses/machine/year while maintaining pregnancy rates. Since 2005 in China, >3.5 million doses of sex-sorted semen have been inseminated into dairy cows to give a 50–60% conception rate per cycle and 94% heifers born.
- Nowadays in the Middle East, camels are used for racing, beauty competitions and meat. The dromedary camel has a short breeding season in the cooler winter months. The female, an induced ovulator, has a long gestation of 13 months but an exceptionally short luteal phase of only 9–10 days. The left uterine horn is bigger than the right and although both ovaries are equally active the embryo always implants in the left horn. The embryo does not enter the uterus from the oviduct until Day 6 after ovulation, yet it must liberate its MRP signal to achieve luteostasis by Day 9, when it is still only a spherical expanding blastocyst. Experiments have shown that neither interferon tau nor embryonic oestrogens (secreted from as early as Day 8) are the MRP factor in camels, the identity of which, as in the horse, remains unknown at the present time. The embryo begins to elongate from Day 10 and implant at Day 14 to produce a diffuse, epitheliochorial placenta with isolated multinucleated giant trophoblast cells dispersed throughout the allantochorion. These secrete oestrogens, the concentration of which rises dramatically in maternal blood during the last 2 months of gestation. The camel ejaculate is small (4–7 ml) and highly gelatinous. Pregnancy rates of 50–55% are achieved when inseminating fresh semen at a dose of 150 million live spermatozoa. However, frozen semen has not yet been demonstrated to be fertile.
- The 'blueprints' of reproduction have varied little within the orders despite major physical alterations and modification over the millennia. This is especially true for the Paeungulata, i.e. the elephant, manatee and hyrax, which share many unusual features of reproduction despite their marked differences in body size and habitat. They all have very long gestation periods, the males have intra-abdominal testes and an ischiocavernous penis of similar shape. The ovaries in the female are encapsulated in a bursa and exhibit multiple corpora lutea throughout pregnancy. They all develop the same type of zonary placenta and show similar hormone profiles during pregnancy. The elephant shows 2 distinct luteinising hormone (LH) peaks at the beginning and end of its follicular phase, only the second of which is accompanied by oestrus, mating, ovulation and conception. Small luteal bodies develop in the unovulated follicles which accompany the first LH peak in the follicular phase. These remain dormant and nonsecretory until the 7th week of gestation when they enlarge greatly and begin to secrete 5α-dihydroprogesterone and other 5α-reduced biologically active progestagens, almost certainly in response to the luteotrophic action of the prolactin secreted by the trophoblast of the implanting embryo at this time. Although accessory corpora luteum (CLs) remain present and active throughout the 22 month gestation, progestagens secreted by the interstitial cells of the greatly enlarged fetal gonads in the second half of gestation likely contribute significantly to maintenance of the pregnancy state.

Conclusion

The presentations recorded above bear witness to the breadth and depth of Professor Twink Allen's contributions to the field of reproductive biology in several species. However, it is in the horse that he has had the greatest impact, through a unique mix of making theoretical advances and ensuring their translation to practical applications to dramatically improve the efficiency of horse breeding throughout the world. The large number of former students and many colleagues from around the world who participated in this Symposium gave testament to the many ways in which Professor Allen has stimulated their research, improved their practice and facilitated contact with like-minded researchers and practitioners.

W. R. Allen, P. D. Rossdale[†], D. F. Antczak[‡] and T. A. E. Stout[§]
The Paul Mellon Laboratory, Newmarket, Suffolk, UK; [†]Romney House, Newmarket, Suffolk, UK; [‡]Baker Institute – College of Veterinary Medicine, Cornell University, Ithaca, New York, USA and [§]Utrecht University - Department of Equine Sciences, Utrecht, The Netherlands

References

1. Antczak, D.F., de Mestre, A.M., Wilsher, S. and Allen, W.R. (2013) The equine endometrial cup reaction: a fetomaternal signal of significance. *Annu. Rev. Anim. Biosci.* **1**, 419-442.
2. Allen, W.R. (2005) The development and application of the modern reproductive technologies to horse breeding. *Reprod. Domest. Anim.* **40**, 310-329.
3. Wilsher, S. and Allen, W.R. (2012) Factors influencing placental development and function in the mare. *Equine Vet. J.* **44**, *Suppl*. **41**, 113-119.
4. Allen, W.R. (2006) Ovulation, pregnancy, placentation and husbandry in the African elephant (Loxodonta africana). *Philos. Trans. R. Soc. Lond. B. (Biol. Sci.)* **361**, 821-834.