WR 'Twink' Allen: A career revolutionising the study and practice of equine reproduction

When Professor William Richard 'Twink' Allen passed away on 6 June 2021, equine reproduction lost its most influential scientist and innovator, and one of its most colourful and inspirational characters. Twink inspired many to enter the field of equine reproduction and encouraged many more to ‘do their bit’ to improve the success of horse breeding, while never losing his own enthusiasm or energy for scientific endeavour. Indeed, he continued to write manuscripts, design experiments, and plan scientific meetings and visits from a ‘post-retirement’ base at Sharjah Equine Hospital up until only weeks before his death, together with his scientific and life partner Sandra Wilsher. Twink’s sudden demise came as a shock to all and meant that he left considerable ‘unfinished business’ in his pursuit of answers to the enigmas of early equine pregnancy. While Twink’s absence will be felt keenly within the world of equine reproduction, he leaves a substantial scientific legacy and it is an honour and privilege to try to put his contribution to equine veterinary science into perspective.

Twink’s extensive bibliography is witness to an immense contribution to equine reproductive science and practice, and also to an unquenchable thirst for knowledge. Equine Veterinary Journal is only one of many journals to which Twink contributed and this month we are documenting Twink’s achievements and contributions to equine reproduction in general and EVJ specifically by releasing a permanent online collection of the articles he has published in Equine Veterinary Journal.

Twink was indefatigable; when he had a new idea or project, he made sure not only that it got done, but that it got done quickly and that the findings were committed to paper in a meticulous and timely fashion so that his work could be of use to others in the research or veterinary communities. In fact, Twink had a passion for writing and for encouraging or coercing others to write; however, he also had high literary standards and was an exacting coauthor and editor who would never allow a manuscript disfigured by grammatical errors or that did not adequately ‘tell the story’ to leave his laboratory. While Twink’s contribution to the literature on equine reproduction can be quantified by the more than 300 papers and book chapters that he wrote or co-authored, his impact as an inspirational presenter, mentor, teacher, and initiator of scientific meetings and collaborations is immeasurable; there are countless veterinarians, scientists and breeders who embarked on a career in horse reproduction as a result of being inspired by one of Twink’s lectures, or performed their first research study or gave their first talk at a scientific meeting after having their arm twisted by Twink over a gin and tonic at a conference social event or ‘gin club’ at the Equine Fertility Unit in Newmarket, where Twink established a world-leading programme in equine reproductive research. While Twink’s name is synonymous with equine reproduction, his interests were much broader, encompassing other areas of equine health and performance, and reproductive physiology and immunology across species, with notable contributions to the reproductive physiology and development of assisted reproductive techniques in camels and to the reproductive biology of African wildlife, in particular the elephant.

Twink’s scientific and academic contributions have been recognised by numerous awards and honours including, but not limited to, a CBE, ScD (Cantab), DSc (h.c. mult.), FRCVS, FRAgSE, FSB and Honorary Doctorates from the Universities of Krakow, Ghent and Helsinki, the Society for Reproduction and Fertility’s prestigious Marshall Medal, and election to the ‘Hall of Fame for Equine Research’ at the Gluck Equine Research Center. Fittingly, one of Twink’s most recent honours was a Lifetime Achievement Award presented at the International Symposium on Equine Reproduction (ISER) in 2018 when the 12th ISER symposium was hosted in Cambridge, 44 years after Twink had initiated and chaired the inaugural ISER meeting, also in Cambridge. The 4-yearly ISER symposia have subsequently become the major international forum for discussing the science and practice of equine reproduction, and the symposium proceedings have been established as an invaluable resource for those interested in keeping up to date with progress in the discipline. Similarly, Twink initiated and hosted the first International Equine Embryo Transfer symposia that continue to be held on a 4-yearly basis ‘mid-way’ between the ISER meetings.

Trying to summarise Twink’s contribution to equine reproductive science is a daunting task, not least because it is much more than simply the sum of his many papers. Moreover, Twink himself eloquently and comprehensively reviewed the major developments in equine stud medicine that he and his peers realised during the last half century for the 50th anniversary edition of EVJ, and he has penned numerous reviews of the physiology of early equine pregnancy and placentaion (eg2,3) and the application of assisted reproductive technologies (ARTs) to horse breeding.4

1 | EQUINE STUD MEDICINE

When Twink finished his PhD on ‘Pregnant Mare Serum Gonadotrophin’ under the supervision of Roger Short at Cambridge University in 1970, most of what today’s equine veterinarian or
breeder would consider as the ‘basics’ in terms of hormone treatments and imaging modalities had not even been thought of. Over the course of 50 years, Twink and colleagues carried out basic and applied studies that changed the diagnostic and therapeutic landscape beyond recognition and, in so-doing, markedly improved both the per cycle and seasonal pregnancy rates in naturally mated (Thoroughbred) mares, and developed and refined the ARTs for widespread application within sport horse breeding. Twink played a major role in translating discoveries in equine reproductive physiology, therapeutic drug discovery and medical technology into equine stud practice, often in collaboration with the two large horse practices in Newmarket. In this respect, he had a gift for understanding what would help tackle the major obstacles to efficient and successful horse breeding, and the drive and powers of persuasion to convince others to help him perform the trials and studies necessary to prove it.

One of the early breakthroughs that Twink enabled, was the application of prostaglandin F2α (PGF2α) analogues to induce luteolysis and return mares to oestrus. This was useful because many mares failed to return to heat at the expected time, thereby seriously reducing the likelihood of successful mating during the relatively short Thoroughbred covering season. While a proportion of the failures to cycle normally were a result of early embryonic loss, the majority were the result of spontaneous persistence of the primary corpus luteum (CL), a condition for which there had previously been no specific treatment. Proof that prolonged dioestrus affected a significant percentage of mares was facilitated by the development of rapid enzyme-linked immunosassays (ELISA) for progesterone that verified luteal activity; PGF2α analogue administration then enabled rapid resolution to salvage the breeding season. One of the other major reasons for mares failing to present to the stallion, was the absence of ovarian cyclicity early in the year, before the natural photoperiodically-stimulated onset of follicle growth; while the most important ‘remedy’ to the man-made problem of an arbitrary covering season that started before nature intended was lighting to artificially shorten the duration of darkness, Twink and colleagues further introduced the use of ‘progestogen withdrawal therapy’. Treating mares with synthetic progestogens, such as alyl trenbolone (now known as altrenogest), for around 10 days helped build up pituitary LH stores to a level capable, after abrupt cessation of progestogen, to rapidly release implants of GnRH analogues that actively stimulated early season follicle growth. Both treatments remain mainstays of early season barren and maiden mare management.

While exogenous hormones markedly improved oestrous cycle management, arguably the single most important technological development in equine stud practice was the introduction of the ultrasound scanner in the late 1970s/early 1980s. First described by Eric Palmer and colleagues in France who adopted the technology from human gynaecology, Twink quickly realised the potential of ultrasonography for the accurate and early detection of pregnancy and, even more importantly, twin pregnancy. The ability to accurately monitor follicle growth, detect ovulation and luteal tissue, visualise uterine oedema, cysts and free fluid and to accurately detect early pregnancy and allow the discrimination and targeted reduction of twins to a singleton has revolutionised equine reproduction; and this is without even mentioning subsequent technological advances that have for example enabled determination of fetal sex and monitoring of fetal and placental development and health. More recent additions to the reproductive veterinarian’s diagnostic and therapeutic armoury pioneered by Twink and colleagues include video-hysteroscopy to examine the uterine lumen and both identify pathology and enable thermic ablation of endometrial cysts and adhesions, and laparoscopic application of PGE2 gel to the oviducts of mares presumed to be suffering from oviducal occlusion; a pilot study on the latter resulted in a remarkably high proportion (14/15) of mares recovering fertility within 1-2 mated cycles, after 1-4 barren seasons. The benefits of introducing these treatments and techniques to the success of Thoroughbred breeding are exemplified via the series of surveys of the fertility of the UK Thoroughbred population carried out by Twink and colleagues. These surveys show a marked increase in per cycle pregnancy rates from around 52% in 1982 to 60%-65% after 1998, and highlight how specified treatments and techniques developed from the mid-1970s onwards have contributed to improved efficiency and enabled an increased number of mares per mating stallion; nevertheless, the surveys also reveal the unremitting impact of early pregnancy loss, given that seasonal foaling rates have improved only modestly from just under 75% to slightly over 80%. This explains Twink’s continued efforts to unravel the processes that ensure maintenance of early pregnancy, or that contribute to embryonic demise.

## 2 | ASSISTED REPRODUCTIVE TECHNOLOGIES

The other area of equine practice transformed by Twink’s work was the development of the assisted reproductive technologies, in particular those involving early embryos. When Twink finished his PhD, horse breeding in the UK was almost entirely performed via natural mating. In this respect, the UK lagged behind other countries that had begun to use artificial insemination (AI) in non-Thoroughbred populations. Surprisingly, the initial major aim of Twink’s first post-doctoral job at the Animal Research Station in Cambridge was to investigate the utility of AI as a tool to rescue Thoroughbred breeding in the case of a novel transmissible disease. Although the Thoroughbred Breeders’ Association quickly pulled the plug on this direction of research, it no doubt prompted Twink and colleagues to publish an overview of the potential pros and cons of AI. In fact, Twink remained a staunch and outspoken advocate of the potential benefits of AI and other ARTs to tackle specific problems in Thoroughbred breeding, invoking the ire of some influential people in the politics of racehorse breeding who did little to prevent the
closure of the Equine Fertility Unit as Twink approached retirement, despite all that Twink's early work had done to help the industry. The cessation of the AI side to his early research may, however, have been a blessing in disguise since Twink was better able to take advantage of being stationed at the world's leading research centre in the development of techniques for, and establishing the limits of donor-recipient synchrony compatible with, successful embryo transfer (ET) in cattle. It meant that Twink was perfectly positioned to perform the first studies on ET in horses. Over the years, the early surgical techniques used to both recover and transfer horse embryos were refined and then replaced by non-surgical methods.

One important early discovery was that, as a result of equine embryo mobility, it was essential to flush the whole uterus of the mare rather than just the tip of the horn ipsilateral to the ovulation. More recently, Twink and colleagues developed a simplified technique for non-surgical transfer using a Polansky type speculum and custom-designed 'Wilsher' forceps to grab the cervix and straighten the cervical canal by pulling; this technique allows the transfer pipette to be passed easily and cleanly into the uterus and makes it possible to achieve high success rates without navigating the steep-learning curve associated with developing the dexterity to successfully perform non-surgical transfers 'manually'. Twink and colleagues also helped better define the acceptable window of donor-recipient synchrony, establishing that horse embryos survive equally well in recipient mares that ovulated between 2 days before and 7 days after the donor; where the former could be extended to 3 days by treating the recipient with meclofenamic acid, while the extremes of negative asynchrony (recipient more than 4 days behind the donor) had the disadvantage of retarding, and potentially compromising, embryo development.

Twink also discovered the incredible ability of equids to accept, and successfully produce offspring, after transfer of embryos from any other equid species (eg between donkey, horse, zebra and Prezwalski’s horse) up to and including the sterile hybrid, the mule. This discovery not only established the potential of interspecies ET in programmes to preserve endangered species but also gave fascinating insights into the physiological and immunological requirements for successful pregnancy. In fact, while most combinations of equids were possible, 80% of pregnancies produced by transferring donkey embryos into pony recipient mares failed between approximately 65 and 100 days of gestation, and the "donkey-in-horse" became Twink’s favoured model for examining aspects of the immunological adaptations required for successful pregnancy maintenance.

Enterprisingly, Twink and colleagues also reported the first long-distance transport of horse embryos, the roughly 1000-mile car journey from Cambridge to Krakow. Six embryos were stored within the ligated oviducts of two rabbits for the 33-hour journey; 5 of these embryos survived and, after transfer to recipient mares synchronised by Polish colleagues, resulted in 3 pregnancies and two live foals. Of course, longer storage durations required cryopreservation, and Twink and colleagues were among those that first adapted slow freezing protocols designed for cattle embryos to horses, and established that only morulae and early blastocysts recovered at day 6-6.5 after ovulation when they were still less than 250 μm in diameter, reliably survived freezing and thawing. Recovering small embryos suitable for freezing was labour intensive (scanning more than once a day), and led to disappointing embryo recovery rates since some embryos were still in the oviduct. Some years later, Twink trialled laparoscopic PGE2 application to the oviduct on day 5 after ovulation to hasten transport of the embryo into the uterus. Although successful, this treatment was invasive and never caught on as a clinical procedure; nevertheless, the accumulations of inspissated follicular fluid, oviductal cells and unfertilised oocytes that often accompanied the prematurely descended embryo gave Twink the idea of using laparoscopic PGE2 application to remedy suspected oviductal occlusion in mares suffering from unexplained infertility. Subsequently, the conundrum of how to reliably cryopreserve the larger horse blastocysts recovered by flushing on day 7 or 8 was solved by the discovery that the key was reducing the blastocele fluid volume by puncture and aspiration; indeed, recent studies by Twink and Sandra Wilsher have helped better define the relationship between the diameter of expanded horse blastocysts and the manipulations required to enable successful vitrification.

Cryopreservation was not the only reason Twink and colleagues wanted to work with very early embryos. Obtaining early embryos, when all or most of the cells were still pluripotent, was essential for the production of genetically identical monozygotic twins. This could be achieved by recovering 4-8 cell embryos surgically from the oviduct and dissecting apart the individual blastomeres, followed by transferring pairs of these blastomeres encaised in an empty pig zona pellucida and embedded in an agar chip, to the oviduct of a recipient mare. More practically, identical twins could be produced by micromanipulator-assisted bisection of morula stage embryos recovered by non-surgical uterine lavage early on day 6 after ovulation, and transfer of the bisected halves to the uterus of separate recipients. Considerable time and effort was spent trying to produce a herd of monozygotic twins as Twink’s ‘ideal matched pairs’ for studies to test pretty much any aspect of (race) horse development and performance but was critically hampered by the inefficiency of actually producing live twins (5%-10%). Nevertheless, the three pairs of identical twin mares at the Equine Fertility Unit subsequently proved their worth as a means to double the speed of generating the material (full-sibling embryos) needed to establish the first comprehensive equine genetic linkage map, an important step on the way to deciphering the horse genome.

Twink was also a pioneer in the more recently introduced ARTs, with his achievements including production of the first pregnancy by intracytoplasmic sperm injection (ICSI) of an in vitro matured oocyte followed by culture of the resulting embryo to the blastocyst stage at which it could be transferred to the uterus; previous studies had either relied on in vivo oocyte maturation or transfer of early post-cleavage stage embryos to the oviduct. Twink correctly predicted that successfully performing all of the steps to blastocyst formation in vitro provided the only realistic basis for a large-scale commercial in vitro embryo production programme. Twink also pioneered the processing of embryos for freezing, and Twink and colleagues were among those that...
development of hysteroscopic insemination as a technique to massively reduce the sperm numbers required for successful AI, thereby paving the way for the use of the very small numbers of sperm that can be separated as reliably X- or Y-bearing within a reasonable time-frame (4-6 hours) by flow cytometry, to achieve pregnancies of predetermined gender. Twink and colleagues were also among the first to establish presumptive equine embryonic stem cell lines, recognising their enormous potential as ‘off-the-shelf’ cell treatments to heal lesions in tissues with poor post-injury capacity for repair and regeneration, such as cartilage and tendon.

3 | PHYSIOLOGY, ENDOCRINOLOGY AND IMMUNOLOGY OF PREGNANCY AND PLACENTATION IN THE MARE

Twink’s remarkable success in driving change and improvement in clinical practice almost masked the fact that he made a number of landmark scientific discoveries. His doctoral work on the origin of the endometrial cups yielded him not only a single author paper in Nature, but led him to discover that the hormone-secreting endometrial cups were embryonic in origin and were generated by an invasive process similar to human embryonic implantation. It also led to the serendipitous discovery of one of the first clear examples of genomic imprinting (parent-of-origin-specific regulation of embryonic gene expression; ie targeted switching on or off of either the paternal or maternal allele to regulate placental development) long before the importance of imprinting and epigenetic regulation were understood; Twink showed that circulating PMSG (now called equine Chorionic gonadotrophin; eCG) concentrations were much lower in mares carrying a mule (donkey father) compared to a normal horse pregnancy, and subsequent comparison of mule, hinny, horse and donkey pregnancies confirmed the importance of paternal genotype in both the size of the chorionic girdle and the resulting maternal eCG concentrations. In fact, much of Twink’s scientific career was devoted to understanding how pregnancy was established and maintained in the mare, and how the embryo/conceptus/foetus communicated with its dam to allow successful implantation, placentation and parturition, and ensured the immunological modulation essential to avoid rejection of the fetal allograft. The ultimate aim was to use understanding of the physiology or immunology, and knowledge of how intrinsic or extrinsic factors could perturb these fundamental processes, to design strategies or treatments to remedy the perturbations and thereby avoid pregnancy loss or damage to the developing foetus. While Twink and colleagues unravelled many of the pieces of the puzzle with regard to how the conceptus and its surrounding blastocyst capsule develop and interact with the uterus and its secretions, and ‘hijack’ maternal physiology to allow implantation and placentation [for review see], to his lasting chagrin the ‘maternal recognition of pregnancy’ signal, ie the embryonic signal that prevents the cyclical demise of the primary CL and thereby ensures the maintenance of the elevated progesterone levels required to maintain early pregnancy, eluded him (and everyone else!); unperturbed and always with a new hypothesis, Twink continued to publish studies on the interactions between the early embryo and its uterine environment into his final year.

Twink was also a pioneer in equine gestational epigenetics. Inspired by the epidemiological studies of David Barker, which focused scientific attention on the critical importance of the intrauterine environment and, in particular, maternal nutrition on post-natal morbidity to adult-onset ‘lifestyle-related’ diseases such as hypertension, ischaemic heart disease and type II diabetes, Twink set about establishing a horse model to test the ‘Barker hypothesis’. He based his model on Walter Hammond’s much earlier study crossing Shire horses with Shetland ponies (by AI) to demonstrate that the equine dam dictated fetal growth and size at birth; ie the foal was much smaller when the mother was a Shetland pony and the father a Shire than vice versa. Twink considered the fact that the monocotyledonary, epitheliochorial placenta of the horse required almost the entire uterine lumen to produce a nutrient-exchange surface large enough to allow a foal to reach its genetic potential in terms of birthweight, to represent the perfect model for examining the effects of manipulating intrauterine growth and nutrition. Embryos were transferred between Thoroughbreds and Welsh ponies to create a standardised model for either growth retardation (Thoroughbred-in-pony) or over-abundant gestational nutrient provision (pony-in-Thoroughbred). As expected, there was a significant effect of maternal size on fetal growth and feto-maternal endocrine function during gestation, and on foal size at birth, subsequent juvenile and adolescent growth and various aspects of post-natal organ and endocrine function. Moreover, the as yet unexplored implication that maternal health and nutrition can affect the health and function of the resulting offspring right through their adult life may well have as yet unrealised significance for the future health and success of competing and recreational horses; yet again, this was an area of study where Twink was ahead of his time.

4 | FINALLY

Unquestionably, Twink Allen was the driving force behind numerous fundamental advances that revolutionised both Thoroughbred and sport horse breeding, to the benefit of the industries involved and the veterinarians tasked with ensuring efficient and successful foal production. And while he was almost certainly ‘one of a kind’, Twink would be the first to champion his good fortune at being ‘in the right place at the right time’; having his embryonic career nurtured by remarkable people who provided expert scientific mentorship and support, lifelong friendship and collaboration, and the right connections within the horse industry. Moreover, he has sowed the seeds for continued progress in equine reproduction via the symposia and meetings that he initiated and the huge number of people that he inspired or encouraged; fuelled by a passion for reproductive science, horses, and a healthy supply of gin and tonic.
REFERENCES


Bibliography: W.R. ‘Twink’ Allen

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EVJ have released this collection of articles by the late W.R. ‘Twink’ Allen to mark his contribution to equine reproductive science

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