Reproduction in Domestic Ruminants VIII

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Preface

The Ninth International Ruminant Reproduction Symposium (9th IRRS) was held at the Hotel Nikko Northland Obihiro in Obihiro City, Hokkaido, Japan on August 25-29, 2014. There were about 170 delegates from 29 countries including participants from Mexico and South America, USA and Canada, Europe, Africa, the Middle East, Australia and New Zealand, and Asia including Japan. The IRRS has been recognized as the most prestigious international conference on domestic ruminant reproduction. The conference is held once every four years, with the most recent conferences held in Anchorage, USA (2010), Wellington, New Zealand (2006), The Crieff Hydro Hotel, Scotland (2002), and Colorado Springs, USA (1998). This was the first time the symposium was held in Asia. The symposium emphasized the most current knowledge and state-of-the-art information on topics relevant mainly to domestic ruminants. Also, it was our consensus that ruminant reproduction directly connects to livestock production worldwide; therefore, we included sessions such as "Pathophysiology and Health in Reproduction," and "Challenges in Optimization of Reproductive Performance".

This volume contains the proceedings of 35 scientific presentations that were made over 14 conference sessions. The 35 invited speakers were selected by the Program Committee that consisted of world-leading experts of each scientific area. The final program included sessions on: Phylogenetics; Genome, Proteomics, Metabolomics; Preimplantation; Neuroendocrinology; Peri-implantation; Challenges in Optimization of Reproductive Performance; Male Function and Spermatogenesis; Oocyte and Follicle; Corpus Luteum; Placentation and Parturition; Pathophysiology and Health; Emerging Reproductive Technology; and Applied Reproductive Technology: Up-date. It also included The Eric Lamming Memorial Lecture on "Physiology of the follicle" in Domestic Ruminants by Bruce Campbell, University of Nottingham, UK.

A conference dinner was held at the "In The Suite" in the City, where the Pioneer Award was made to Professor Kenneth P. McNatty, Victoria University of Wellington, NZ, for his outstanding contributions to our understanding of Ruminant Reproduction.

We gratefully acknowledge the financial support of Zoetis Japan, Intervet, Zenrakuren, Ohtsuki Physics and Chemistry, Fijihira Industry, Japan Sugar Beet Industry (Nitten), Animal Genetics Japan, Hitachi-Aloka Medical, Genetics Hokkaido, Society for Reproduction and Development, Society for Reproduction and Fertility, Society for Reproductive Biology, American Society of Animal Science, Brazilian Embryo Technology Society, Obihiro City, Hokkaido Prefecture, and Obihiro University of Agriculture and Veterinary Medicine.

The organizing committee consisted of Akio Miyamoto (Obihiro, Japan), Mike Smith (Missouri, USA) and Bob Webb (Nottingham, UK), who co-chaired the conference and the editing of the proceedings. In addition, we had an excellent and professional team for editing the 35 invited papers as well as 90 abstracts of poster presentations with Jenny Juengel, Chris Price and Larry Reynolds. The authors of 35 invited papers made an outstanding effort to finalize their papers, and thus we were able to complete the editing this book before the conference took place, so that all participants could have the proceedings in hand at the conference in Obihiro.

This volume could not have been competed without the highest quality work by Sarah Keeling (Context Publishing), who assembled and typeset these proceedings. Special thanks go to the members of the Program Committee, chairpersons of the sessions, and all the reviewers of invited papers and abstracts of poster presentations. Finally, a special thank you goes to Moto Matsui (Obihiro University), Secretary General, who helped me and worked on all the issues for local arrangement and managing the conference. This symposium would not have been possible without any of these volunteers. I believe the whole effort will provide a strong example to to the organizers of the next International Ruminant Reproduction Symposium organizers.

Akio Miyamoto
Chairman, 9th IRRS

Ruminant phylogenetics: A reproductive biological perspective

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Summary

Phylogenetics is the study of evolutionary relationships among species. Phylogenies are based on the comparison of large numbers of characteristics among species. Traditionally, the field of phylogenetics was dominated by paleontologists so the characteristics studied were structural, often skeletal. The field of phylogenetics was revolutionized in the 1980s as scientists began using molecular data, first amino acid, then nucleotide sequences. This led to the inclusion of more characteristics and many more extant species in these analyses. We now have very well characterized phylogenies for most major groups of mammals, including the ruminants (*Ruminantia*, a suborder within *Artiodactyla*). The ruminants are traditionally divided into six families: *Tragulidae* (mouse deer), *Moschidae* (musk deer), *Cervidae* (true deer), *Antilocapridae* (pronghorn), *Giraffidae* (giraffes and okapis) and *Bovidae* (horned ruminants). Despite extensive research, some phylogenetic relationships within the *Ruminantia* have not been completely resolved. For example, the precise relationships among the six ruminant families is not clear. The relationship of cattle (*Bos taurus*) to other large bovids (gaurs, bison, yaks, etc.) remains to be determined. Ultimately, more extensive characterization and comparison of ruminant genomes will define these relationships. In the mean time, we may be able to use reproductive characteristics to help clarify some of the unresolved phylogenetic relationships. Reproductive characteristics can vary greatly among species. Much of this variation is recently evolved, making it particularly useful in defining relationships among closely related species or groups. Placentally expressed gene families, reproductive behaviors and even interspecies embryo transfer studies may provide novel ways to resolve the few remaining phylogenetic questions in ruminants. Recognizing that the vast majority of existing phylogenies are extremely accurate, reproductive biologists can use them to make more rapid progress in extending research from one species to another. Phylogenies also can provide a background to determine how specific reproductive characteristics evolved over time. Finally, phylogenetics and reproductive biology can be brought together to study the fundamental biological process of speciation. Speciation is the study of how new species arise. Establishing reproductive isolating barriers (variation) between a nascent species and its immediate ancestor is a fundamental part of the speciation

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process. Much of the work in this area has been done using invertebrate species with very short generation intervals. Mammalian models to study speciation are severely lacking. Ruminants may be an ideal group in which to study this process since they have the two prerequisites essential to this type of work, 1) a large number of recently-evolved extant species and 2) well characterized and dated phylogenies. The body of fundamental research characterizing reproductive systems in a few ruminants is enormous. We are at a point where we can start to extend more of this research to other ruminant species to address the process of speciation, and perhaps other, fundamental biological questions.

What is phylogenetics?

Phylogenetics is the study of evolutionary relationships among species. Phylogenetic relationships are proposed based upon thorough, systematic and on-going comparisons of diverse characteristics. Thus, they are under continuous revision. Scientists had been classifying and organizing species based on the comparison of morphological characteristics since the 1700s. These types of comparisons were the basis for the first comprehensive taxonomic classifications by Carl von Linné (Linnaeus), Georges Cuvier, Richard Owen and others. To be clear, these authors were only using the comparisons to establish associations for classification. For example, sheep, goats and cattle have two toes on each foot and thus belong in the same taxonomic "box". Horses, having just a single toe on each foot, belong in a different "box". These authors never intended to imply that sheep, goats and cattle were more closely related to each other than they were to horses. It was Owen who first used the terms '*Artiodactyla*' and '*Perissodactyla*' in referring to the even-toed and odd-toed ungulates, respectively. One of the earliest and most comprehensive taxonomic classifications of mammals was published by Gray (1821) in which the ruminant families *Moschidae, Giraffidae* and *Bovidae* were first formally named.

The first true 'phylogenetic tree' , one in which descent from common ancestors was implied, was drawn by Charles Darwin and published in Chapter IV of his *Origin of Species* (1859). Traditional taxonomic classification was almost immediately reconsidered with an evolutionary perspective. It is interesting to note that very little revision of the traditional taxonomic classification was required. The morphological traits upon which the taxonomies were based were essentially the same ones that were used to establish evolutionary relationships. Subsequently, more detailed analyses of both skeletal and soft tissue morphology were conducted. More recently, behavioral (Tinbergen, 1959; Lusseau, 2003) and embryological (Hall, 2000) characteristics have been considered in phylogenetic analyses. Such efforts have been augmented by the inclusion of more species and by increasingly rigorous evaluation of individual variation within species. Finally, mathematical methods for constructing phylogenies were developed and improved (Camin and Sokal, 1965; Felsenstein, 1985). Mammalian phylogenies underwent a period of regular revision, but eventually, a general consensus emerged. Simpson (1945) published a mammalian phylogeny that represents this consensus very well into the 1980s.

What are ruminants?

Ruminants are the most numerous group of extant ungulates (hoofed mammals). There are at least 250 recognized species of ruminant. The phylogenetic grouping *Ruminantia* is a suborder

(McKenna and Bell, 1997) within the mammalian order *Artiodactyla*. Ruminants can be distinguished from other mammals by a few unique morphological characteristics. The cuboid and navicular bones in the tarsus are fused in all ruminants, including the oldest known fossil forms (ex. *Dorcatherium*; Milne-Edwards, 1864; *Hypertragulus*, *Archaeomeryx* and *Gelocus*; Webb and Taylor, 1980). Ruminants are one group among many groups of mammals that have compartmentalized stomachs to facilitate microbial digestion. However, ruminants can be distinguished from these other groups by two unique gross morphological characteristics of their compartmentalized stomach. The first is the structure of the first stomach compartment, the rumen. As in most mammals, the esophagus in artiodactyls joins the stomach along the lesser curvature. This leaves a blind sac at the 'anterior' end called the fundus. The rumen develops embryologically as an elongation of the fundic region. In ruminants, this elongated fundic 'sac' undergoes a unique, secondary folding into a Z-shaped pattern during development. The external surfaces of the developing 'rumen' fuse where they are brought into contact by the folding, creating the rumen's unique internal architecture (Hofmann, 1973; Langer, 1974; Stevens and Hume, 1995). It can be distinguished easily from the foregut structure of all other mammals, including other artiodactyl foregut fermenters, like camels, hippos and peccaries (Vallenas et al., 1971; Langer, 1975; Schwarm et al., 2010). Another unique structural feature is found in the second stomach compartment, the reticulum. The ruminants have a reticular network of ridges lining the inside surface of the reticulum (Hofmann, 1973). This is what gives the inside surface of the reticulum its characteristic "honeycomb" appearance. These features define ruminants to the exclusion of any other species.

The modern ruminants are divided into six families. The six families are presented in Table 1 along with three of the morphological characteristics that define them. The first characteristic is facio-cranial ornamentation, the presence of pronounced structures on the face or cranium that are used primarily in intraspecies competition for mates. These are typically sexually dimorphic, more highly developed in males, less developed or absent in females. In ruminants, tusks are well developed upper canines and are the only facial form of ornamentation. Cranial forms include horns, antlers and ossicones. Another distinguishing characteristic is the omasum, a compartment of the stomach that can sit between the reticulum and the last compartment, the abomasum. The omasum has 50-100 longitudinal folds of tissue suspended from the inner surface of its greater curvature. They lay parallel to each other creating a very effective filtering mechanism that greatly reduces the rate of passage of digesta into the abomasum. The last distinguishing characteristic in Table 1 is the form of placentation. The ruminant families differ in the form of placentation, based on the distribution of chorionic villi. These patterns of distribution can be diffuse or cotyledonary (associated with uterine caruncles). The cotyledonary form can be either oligocotyledonary in which there only 6-10 large uterine caruncles and associated cotyledons or polycotyledonary in which there are more than 50 small uterine caruncles and associated cotyledons.

Table 1. The families within *Ruminantia* and three of the morphological characteristics that help define them.

FAMILY	Number of species	Facio-cranial ornamentation	Omasum	Placentation
Tragulidae	10	tusks	no	diffuse
Moschidae	7	tusks	yes	oligocotyledonary
Cervidae	90	tusks or antlers	yes	oligocotyledonary
Giraffidae	2	ossicones	yes	polycotyledonary
Antilocapridae	1	horns (deciduous)	yes	polycotyledonary
Bovidae	140	horns (permanent)	yes	polycotyledonary

The *Tragulidae* (mouse-deer, chevrotains) separated from the rest of the ruminants about 35 million years ago (Bibi, 2013). Only ten species of tragulid survive today. Their geographic range is limited to tropical forests in Asia and Africa. They are the least derived of the six families. In other words, they have probably changed the least from the last ancestor common to all ruminants. Some consider tragulids to be 'living fossils' (Rössner, 2007). Among the ancestral characteristics maintained in tragulids are 1) tusks as opposed to cranial ornamentation (ossicones, horns or antlers), 2) the lack of an omasum and 3) a diffuse placentation. The remaining five families of ruminants are collectively referred to as the *Pecora* (McKenna and Bell, 1997) or pecoran ruminants. All pecorans have omasa. The *Moschidae* (musk deer) are among the least studied of the ruminant families. There are only seven known species of moschid. They are only found in temperate forests of Asia. They have retained tusks but are easily distinguishable from tragulids by having a well-developed omasum and being oligocotyledonary. The *Cervidae* are the true deer. There are about 90 species within the family *Cervidae*. They can be found in a variety of habitats (arctic to tropical) throughout Eurasia and the Americas. Most cervid species have antlers. Antlers are a unique form of cranial ornamentation. They consist of a dense, often branching, core of cartilaginous tissue that grows and gradually ossifies. During the growth phase, the antler is covered with skin (velvet). Once fully ossified, the skin is shed, leaving the completely ossified antler exposed. At the end of the breeding season, the antlers are shed. For this reason, antlers are often referred to as being 'deciduous'. A new set is grown each year (Davis et al., 2011). As a rule, the upper canines are absent in antlered cervids. There is one prominent group of cervids, the Chinese water deer (*Hydropotes*), that does not have antlers. They have well developed tusks instead. Just to complicate things further, elk (both the Eurasian (*Cervus elaphus*) and North American (*Cervus canadensis*) forms) have well developed antlers and also express small, upper canine 'tusks' in both sexes. Like the moschids, cervids have an omasum and an oligocotyledonary placentation. While many species of cervid are raised in captivity, reindeer are the only cervids that can be considered domesticated (Clutton-Brock, 1981). Due to their economic value (antler, meat, hide), there has been a fair amount of research done with cervids. *Bovidae* is the ruminant family with the greatest number of species. The family includes all of the Asian and African antelopes and gazelles. It also includes the truly domesticated ruminants: cattle, sheep, goats, water buffalo and yaks (Clutton-Brock, 1981). Bovids are found naturally in a wide range of habitats throughout Eurasia, North America and Africa. The domesticated forms have been distributed in large numbers to every part of the globe, including areas like South America, Australia, New Zealand where bovids never existed naturally. Owing to their large numbers and economic importance, the domesticated bovids are the most well studied ruminants. They are polycotyledonary and have true horns. In *Bovidae*, a horn consists of three parts, 1) a boney horn core, 2) a layer of specialized skin over the horn core and 3) a keratinized sheath covering the skin. The horn core grows continuously, throughout the animal's life. The keratinized sheath also grows to accommodate the core (Davis et al., 2011). The *Giraffidae* is a small family with only two extant species, the giraffe and okapi. They are geographically limited to the plains and forests of Africa, respectively. They are characterized by ossicones, a unique form of cranial ornamentation. Like horns, ossicones have a bony horn core that is covered with skin. Unlike horns, ossicones do not have a keratinized sheath (Davis et al., 2011). *Giraffidae* are polycotyledonary. The family *Antilocapridae* includes a single species, the pronghorn antelope of North America. The distinguishing feature for this family is the deciduous nature of its horn sheath. As in the *Bovidae*, the horn core grows throughout the animal's life. However, pronghorns shed the keratinized sheath each year as a new sheath is grown beneath to replace it (Davis et al., 2011). Like the giraffids, pronghorns are polycotyledonary.

Recent advances in mammalian phylogenetics that impact ruminants

Over the last 30 years, mammalian phylogenetics has undergone some remarkable revisions. This has been due to the application of modern gene-sequencing technology, permitting the comparison of mRNA and DNA sequences among species. Two of the most intriguing revisions have occurred within the order *Artiodactyla* and have important implications for the ruminants. The first involves the relative position of the family *Camelidae* (camels and llamas). *Camelidae* had traditionally been considered sister taxon (the closest relatives) to the ruminants. This was based on highly-derived features shared by the two groups. These include the loss of upper incisor teeth, a compartmentalized stomach (three compartments in camelids), the reduction in the number of digits in each foot to two and the evolution of 'rumination' as an obligatory physiological process. A phylogenetic tree showing the popularly held view of relationships among families within the order *Artiodactyla* is shown in Figure 1. These relationships were widely accepted into the 1980s. Modern nucleotide sequencing data now clearly shows that the *Camelidae* was the earliest major branch to diverge from the stem artiodactyl group (Graur and Higgins, 1994; Gatesy, 1997; Gatesy et al., 1999; Shimamura et al., 1999; Spaulding et al., 2009). This divergence occurred about 60-65 million years ago (Bininda-Emonds et al., 2007; O'Leary and Gatesy, 2008; Hassanin et al., 2012). Despite

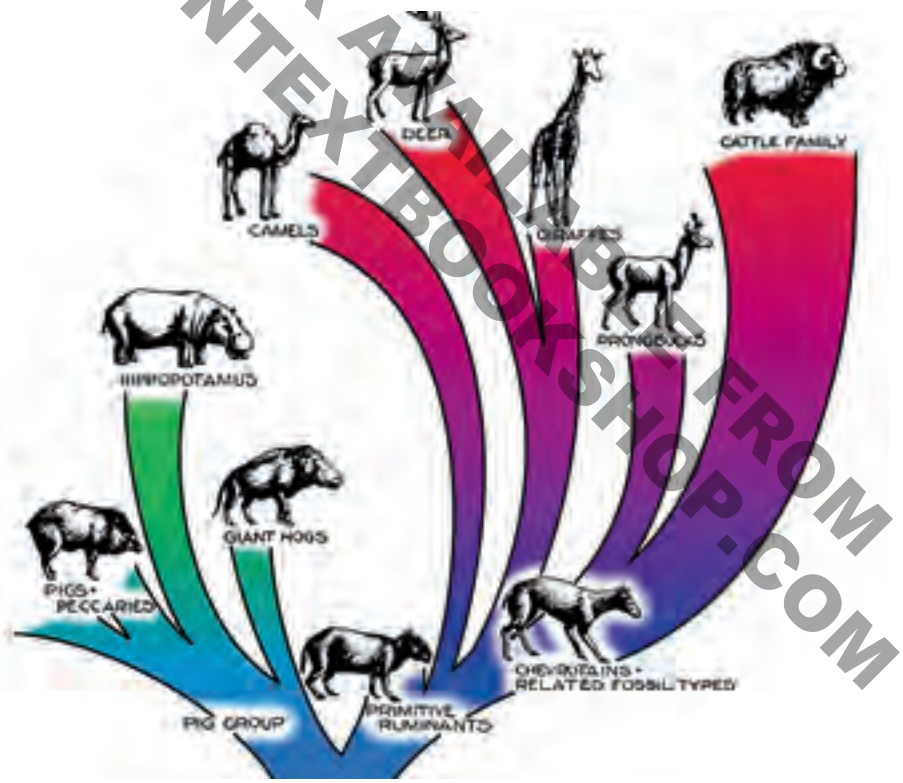

Figure 1: A family-level, phylogenetic tree of the *Artiodactyla* that is representative of the popular consensus circa 1930-1980 (Romer and Parsons, 1977).

Original caption: A family tree of the even-toed ungulates (artiodactyls). The major cleavage is into the pig group (left) and the cud-chewers, or ruminants (right). Among the latter, the camels appear to have diverged at an early date.

relationships remain unresolved. As described in the second section, the *Tragulidae* are an ancient family separating from the rest of the ruminants 30-35 million years ago . Precise phylogenetic relationships among the remaining five ruminant families have not been resolved. This is due, in large part, to the small numbers of extant species left in many of the remaining families. There is no doubt that all five of these families diverge from each other between 17 and 25 million years ago (Bibi, 2013). The problem is in defining the precise pattern and order. Recent studies, with larger data sets, place the *Giraffidae* and *Antilocapridae* as early offshoots from the ruminant tree (Decker et al., 2009; Spaulding et al., 2009; Hassanin et al., 2012; Bibi, 2013). The *Moschidae* are consistently found to be closer to the *Bovidae* than to the *Cervidae*. Within families, most phylogenetic relationships are well defined. This is particularly true for the families with 10 or fewer extant species. Within the more numerous *Bovidae* and *Cervidae*, some phylogenetic relationships have yet to be resolved.

Before describing the phylogenetic relationships within specific ruminant families in detail, it is important to address how phylogenetic trees are determined. This is explained briefly

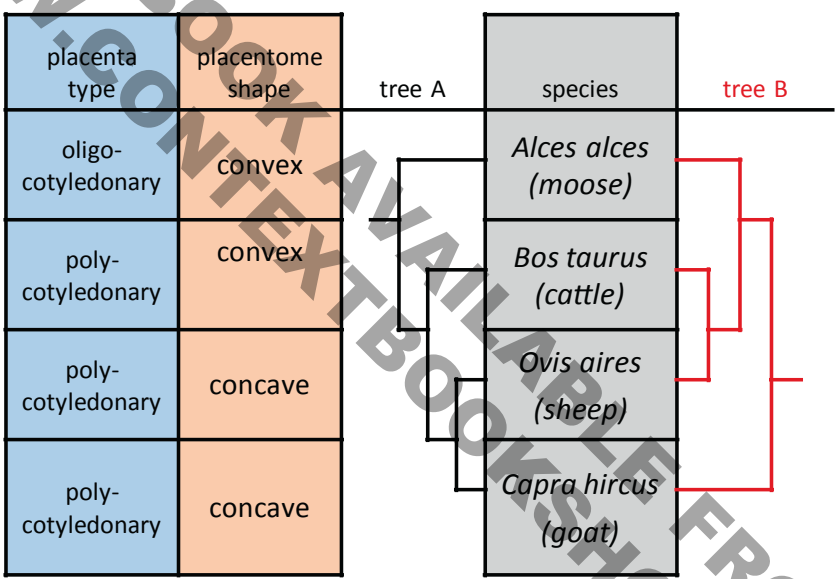

Figure 4. Building a phylogenetic tree. A phylogenetic tree is an attempt to depict the simplest set of evolutionary relationships that can explain the distribution of a set of characteristics among a group of species. Phylogenetic trees can be generated using a variety of different mathematical approaches. Each of these methods attempts to find the tree that minimizes transitions from one character state to another. The assumption is that specific evolutionary changes are rare, unlikely to repeat themselves and very unlikely to reverse themselves. For comparisons involving morphological characteristics, reversals are almost impossible. For comparisons involving nucleotide sequences, reversals are certainly rare but possible. In the very simple example shown here, we have four species and just two placental characteristics. Assume that the oligocotyledonary condition is the ancestral (starting) condition. The polycotyledonary condition only has to 'evolve' once, in a common ancestor to cattle, sheep and goats. Assume that the convex shape is ancestral. The concave placentome only has to 'evolve once', in a common ancestor to the sheep and goat. Any other tree will require more transitions. Tree B is an alternative. Once again, assume that the oligocotyledonary condition is ancestral, then the polycotyledonary condition evolved independently, at least twice, in the line leading to the goat and again in a common ancestor to sheep and cattle. Likewise, if the convex placentome is ancestral, the concave placentome evolved twice, in the line leading to goats and again in the line leading to sheep. Thus, tree B is a much more complicated explanation for how these characteristics evolved than tree A. While tree B is certainly a possible explanation and may actually be true, tree A is much simpler and much more likely to be true.

in Figure 4. It is also important to understand how to interpret the information presented in trees and to evaluate their accuracy. This is described in Figure 5. The major phylogenetic relationships within the *Bovidae* are presented in Figure 6. *Bovidae* are traditionally separated into eleven 'tribes'. Species from two of these tribes (*Caprini* and *Bovini*) have been successfully domesticated. There are about 35 species in the *Caprini*. A phylogeny for a representative group of these is presented in Figure 7. Domestic sheep and goats are both members of the *Caprini*. A proposed phylogeny for the *Bovini* is presented in Figure 8. Domesticated members

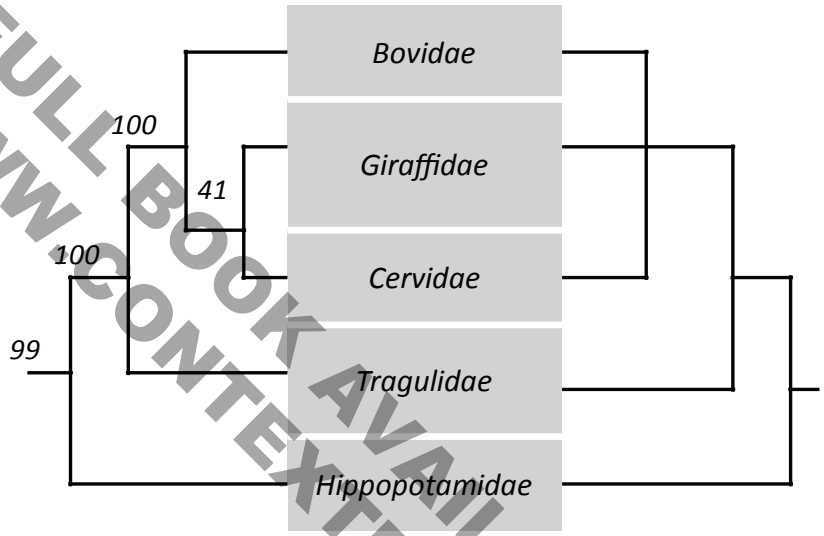

Figure 5. Reading and evaluating a phylogenetic tree. This is a portion of the phylogenetic tree presented by Gatesy (1997). It shows the phylogenetic relationship among four families within *Ruminantia* and the Hippopotamidae. In the orientation used here, it may be simplest to move backwards in the evolutionary sense (to the left), from the individual species. The phylogenetic tree indicates that the ruminant families *Giraffidae* and *Cervidae* are more closely related to each other than to any other families in the analysis. In some trees, the length of the horizontal lines leading to the junction point is representative of the number of character differences between the groups (or species). Keep in mind that we are moving in evolutionary 'reverse'. If we were to follow the evolutionary path, the 'junction point' is really a divergence point, where groups separate. In many trees, a numerical value (0-100) can be found at the junction points. These are bootstrap values. These are estimates of how reliable the association between these groups is. Bootstrap values are generated by rerunning the tree forming algorithm using a subset of the starting data set in which only some of the characteristics, chosen at random, are used. These tests are usually done 500-2000 times. The bootstrap value is the % of these trees in which this relationship is supported. In this case, the bootstrap value is 41%. This is very low. Most phylogeneticists use 80% bootstrap support as a cut off for a supported relationship. Thus, we have little support for the association between *Cervidae* and *Giraffidae*. Alternative associations (*Bovidae-Giraffidae, Bovidae-Cervidae*) are very possible. Presentation of this relationship in a tree can take two forms. The weakly supported relationship is depicted on the left. The alternative is to present the unresolved relationship as on the right, as several lines coming together at a single junction point or level. This implies that the specific relationships among groups at this level have not been resolved. The relationships are much clearer at subsequent levels. Continuing to the left, the bootstrap value for the group of *Bovidae-Giraffidae-Cervidae* is 100%, indicating that this group of three consistently clusters in every tree. Likewise, the association with the *Tragulidae* consistently occurs at the next level. The last family included in this tree is *Hippopotamidae*, an outgroup. Every phylogenetic tree should include at least one outgroup for comparison. An ideal outgroup is phylogenetically close to, but clearly outside of, the group being analyzed. In this case, *Hippopotamidae* is an artiodactyl, but not from the suborder *Ruminantia*. It is less constructive or informative to use very distantly related species as outgroups (ex. canids, primates, birds) as these rarely challenge the characteristic data set on which the group is being evaluated.

Embryo cryo-preservation by vitrification for implementation of bovine embryo genomic selection

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The combination of genetic selection and assisted reproductive technologies can increase the intensity of selection and accelerate the rate of genetic gain in dairy cattle[1]. One strategy for maximising genetic gain is through the production of embryos from genetically superior parents using *in vitro* fertilisation (IVF). To manage the logistics of embryo genomic selection, the cryo-preservation of embryos is critical after biopsy and before transfer of selected embryos to surrogate cows for the birth of spring-born calves. Vitrification is a cryo-preservation procedure that involves ultra-rapid cooling in a concentrated cryo-protectant solution. As it bypasses ice crystal formation, vitrification may reduce the incidence of cryo-damage to embryos compared to conventional slow-freezing. In a preliminary trial using abattoir-derived oocytes, vitrification protocols based on the Cryologic vitrification method[2] were refined to achieve rates of *in vivo* development for biopsied Day 7 IVF embryos that were similar to fresh control embryos on Day 65 of gestation (18/42 = 43% vs. 16/42 = 38%)[3]. In the study here, we report on the pregnancy rates and development to term of biopsied and vitrified IVF embryos, produced using oocytes recovered via trans-vaginal ovum pick-up, in a proof-of-principle trial investigating embryo genomic selection.

Ovum pick-up was performed once per week on one elite cow over 14 weeks. Oocytes were fertilised with sperm from a single elite sire and a total of 62 full-sib blastocysts were produced that were suitable for biopsy on Day 7 of development. Following biopsy of the trophectoderm using a micro-surgical blade, the remainder of each blastocyst-stage embryo was individually vitrified and stored in liquid nitrogen. The genomic DNA from each of the previously frozen embryo biopsy samples was amplified to provide sufficient material for genotyping using the Illumina 50K BeadChip[3]. The resulting single nucleotide polymorphism (SNP) genotypes were then used for the calculation of estimated genomic breeding values for each embryo. Selected male and female embryos were warmed and a total of 32 embryos transferred singularly to the *uteri* of synchronised recipient heifers. In addition, 10 fresh IVF embryos, produced from the same dam and sire combination, were transferred as controls. On Day 100 of gestation, there was no difference in pregnancy rates between the biopsied/vitrified embryos used for genomic selection and the fresh control embryos (53% vs. 30%, respectively). Importantly, there were no pregnancy losses up to full term. In the biopsied/vitrified embryo group, 15/18 calves born survived the immediate post-natal period, compared to 3/3 calves derived from the control embryos.

In conclusion, vitrification of Day 7 biopsied bovine embryos offers a viable method for storing embryos to manage the logistics of embryo genomic selection in dairy cattle breeding schemes.

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[1] Ponsart *et al*., 2014. Reproduction, Fertility and Development 26: 12-21.
[2] Fry *et al*., 2005. Reproduction, Fertility and Development 17: 272.
[3] Fisher *et al*., 2012. Proc. N.Z. Soc. Anim. Prod.72: 156-158.

Effect of breed on embryo production in superovulated ewes

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The efficiency of an embryo transfer program can be affected by many factors including genotype of the ewe[1]. The objective of this study was to evaluate the effect of ewe donor breed on ovulation rate, embryo recovery rate, and number of transferable embryos in superovulated ewes.

Fifty-one ewes from three breeds (24 Dorper, 18 Charollais, and 9 East Friesian) were superovulated. Ewes were synchronized with intravaginal sponges containing 40 mg of fluorogestone acetate (FGA; Chronogest, Intervet) inserted for 12 d followed by 75 µg of prostaglandins (Prosolvin, Intervet) per donor administered on d 10 (the day of sponge insertion was considered d 0). Follicle stimulating hormone for superovulation was administered every 12 h during 4 d through 8 intramuscular injections in a descending protocol (50, 50, 40, 40, 30, 30, 20 and 20 mg, respectively). The treatment began on d 10, 60 h before sponge removal and finished 24 h after. The ewes were inseminated 20 h after onset of estrus through laparoscopy with 200 x 10^6 spermatozoa as fresh semen from a ram of the same breed of the donor and with known fertility. Embryo recovery was performed on d 7 after estrus and ovulation rate was determined through mid-ventral laparotomy at the same time. The embryos were evaluated taking into consideration morphological characteristics. Data were analyzed by ANOVA or logistic regression as required.

There were differences ($P < 0.05$) in ovulation rate between Charollais and Dorper ewes (18.2 $\pm$ 0.99 vs. 10.7 $\pm$ 0.86, respectively), but there were no differences ($P > 0.05$) between Charollais and East Friesian ewes (18.2 $\pm$ 0.99 vs. 14.4 $\pm$ 1.41, respectively) or between East Friesian and Dorper ewes ($P > 0.05$; 14.4 $\pm$ 1.41 vs. 10.7 $\pm$ 0.86, respectively). There were no differences ($P > 0.05$) in embryo recovery rate between the three breeds (Charollais, 75%, Dorper, 63%, and East Friesian 56%). The number of transferable embryos was greater ($P < 0.05$) in Charollais (12.8 $\pm$ 1.2) compared to Dorper (4.6 $\pm$ 1.0) and East Friesian (7.6 $\pm$ 1.6) ewes.

In conclusion, the results showed differences between breeds in the response to the superovulatory treatment administered.

[1]Vivanco *et al*., 1994. Theriogenology 41(1): 329 (Abstract).

Time of change of medium in a sequential medium culture and early development of sheep embryos *in vitro*

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The efficiency of *in vitro* embryo production systems is generally low; therefore, the present study had the objective to evaluate the optimum time to change the culture medium in a sequential medium system.

Grade A oocytes (n = 1,352) were obtained from ovaries collected from ewes slaughtered at a local abattoir. The maturation, fertilization, and culture were carried out in a CO_2 incubator maintained at 38.5 °C, with 5% CO_2 in air with maximal humidity. The oocytes were matured in TCM-199 medium supplemented with 10% fetal bovine serum, 5 μg/mL FSH, 5 IU/mL hCG, and 1 μg/mL estradiol-17ß. The medium was covered with 200 μL of mineral oil, and the oocytes were incubated for 24 h. After maturation, the oocytes were fertilized in TALP-HEPES medium with fresh capacitated semen (250,000 spermatozoa contained in 4.5 μL per well) and were incubated for 18 h. The resultant eggs were cultured in one of three sequential media systems: T1 (n = 414) cleavage medium for the first 48 h and blastocyst medium for the last 120 h of culture, T2 (n = 421) cleavage medium the first 60 h and blastocyst medium the remaining 108 h, and T3 (n = 517) cleavage medium the first 72 h and blastocyst medium the following 96 h. The results were analyzed using ANOVA or logistic regression as required.

The embryo development up to morulae was different ($P < 0.05$) among the three treatments (60.63 vs. 66.92 vs. 73.39% for T1, T2 and T3, respectively). The development up to blastocysts was greater ($P < 0.05$) in T2 compared to T1 and T3 (32.54 vs. 13.77, and 16.63%, respectively). The size of the embryos determined at the stage of compact morulae was only different ($P < 0.05$) between T2 and T3 (149.60 $\pm$ 8.5 vs. 145.56 $\pm$ 7.8 μm), whereas T1 ($P > 0.05$; 146.8 $\pm$ 6.1 μm) was similar to T2 and T3. There were no differences among treatments ($P > 0.05$) in the morphological quality of the embryos.

In conclusion, the optimum time to perform the change of culture in the sequential culture medium used was at 60 h under the conditions of our study.

Meta-analysis of recombinant bovine somatotropin-Zn (rbST-Zn) on reproductive responses in lactating dairy cows

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The commercial form of recombinant bovine somatotropin, sometribove zinc formulation (rbST-Zn), was approved by FDA as safe and has been successfully used by the U.S. dairy industry since 1994. The present investigation utilized a series of meta-analyses to re-evaluate the efficiency and safety of rbST-Zn when used according to label. A total of 26 studies, involving a total of 13,784 cows, met the criteria which were: 1) published in peer-reviewed journals or reviewed by regulatory agencies, 2) used the rbST-Zn formulation (Posilac) available to US producers, and 3) use was according to label for dose (biweekly), treatment initiation (57-70 d postpartum), and administration method (subcutaneous injection).

For the first two controlled breeding cycles after the voluntary wait period, meta-analysis (9 studies) detected a 5.4% improvement in pregnancy proportion (PP) in response to rbST-Zn (29.1% control $<$ 34.5% rbST-Zn; $P < 0.01$). However, a 5.5% decrease ($P < 0.05$) in PP was detected during the accumulated length of the studies (6 studies; 76.1% control $>$ 70.6% rbST-Zn; $P < 0.01$). The two studies with the greatest weight in the meta-analysis due to large sample size had minimal changes in accumulated PP for rbST-Zn of -4.4% and +2.3%. There was no effect of rbST-Zn on fetal loss (11.5%, $P < 0.65$; 9 studies), days open (104, $P < 0.96$; 5 studies), services per conception (1.66, $P < 0.12$; 4 studies), twinning (6.5%, $P < 0.68$; 2 studies), or cystic ovaries (6.5%, $P < 0.68$; 3 studies). Meta-analysis results indicated that rbST-Zn increased 3.5% fat-corrected milk yield (4.04 kg/d, $P < 0.001$; 13 studies). The rbST-Zn reduced body condition scores (1 to 5 scale) (0.06 point, $P < 0.03$; 15 studies), a difference in body weight of about 3 kg.

When used according to label, rbST-Zn had no significant detrimental effect on reproductive responses that were evaluated, including occurrence of pregnancy during a defined limited experimental period at initiation of rbST-Zn treatment, days open for pregnant cows, number of services required per conception, occurrence of fetal losses, incidence of twinning and occurrence of cystic ovaries. A decrease in accumulated pregnancy response over the entire experimental period was likely attributable to reduced occurrence of cows detected in estrus and presented for insemination, due to the well-established increase in milk production and metabolism following treatment with rbST-Zn. The temporal increase in PP detected within a defined reproductive management program, following on-label injection of rbST-Zn, is consistent with rbST exerting an overall complementary reproductive effect with milk production, on productivity and well-being of dairy cows.

The quality of blastocysts produced from in vivo and in vitro matured Holstein oocytes by in vitro fertilization with X-sorted sperm

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Recently, both in vivo and in vitro matured oocytes obtained by ovum pick up (OPU) after superstimulation in Holstein cows have been successfully used for the production of female embryos by in vitro fertilization (IVF) using X-sorted sperm. It is generally accepted that in vitro matured oocytes have lower competence for embryo development compared to their in vivo matured counterparts. However, the quality of blastocysts in terms of cell numbers, DNA fragmentation and freezing tolerance remained unknown. The aim of the present study was to compare the quality of blastocysts obtained by IVF with X-sorted sperm and in vivo and in vitro matured bovine oocytes with that of in vivo-derived embryos.

In vivo-matured oocytes were collected from ≥ 5-mm follicles by OPU from Holstein cows (n = 24) just before ovulation after superstimulation (group A). Immature oocytes were collected from ≥ 2-mm follicles of Holstein cows (n = 10) by OPU without hormonal treatment and matured in vitro (group B). Oocytes were inseminated with X-sorted Holstein sperm and cultured for 9 d. A random selection of d 7 to 8 blastocysts derived from groups A (n = 86) and B (n = 24) were examined as detailed below. In vivo-derived embryos were collected from superstimulated Holstein cows on d 7 after insemination (Day 0 = estrus; group C, 37 embryos, 8 cows). Cell numbers and DNA fragmentation were evaluated in blastocysts by differential staining of ICM and TE cells combined with TUNEL staining. For the evaluation of cryotolerance, blastocysts were preserved by conventional slow freezing with 1.36 M glycerol and 0.25 M sucrose, then thawed and cultured for 48 h.

Higher numbers of ICM, TE and total cells were recorded in blastocysts of groups A and C than group B (ICM: 39.6 and 43.5 vs. 24.5; TE: 81.0 and 86.4 vs. 58.5; total: 120.6 and 129.9 vs. 83.1, respectively; $P < 0.05$). There was no difference in the TUNEL-positive TE and total cells among groups; however, the TUNEL-positive ICM was lower in groups A and C compared with group B (8.2 and 7.9 vs. 11.5 % of cells, respectively; $P < 0.05$). Rates of embryo survival and hatching and hatched blastocysts at 48 h of culture after thawing in groups A and C were higher than in group B (survival: 90.3 and 88.2 vs. 57.1 %; hatching and hatched: 74.2 and 88.2 vs. 57.1 %, respectively; $P < 0.05$).

In conclusion, embryos produced from in vivo matured oocytes by IVF with sex-sorted sperm resembled in vivo-derived embryos and were superior to those generated from in vitro matured oocytes.

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