SESSION 2

EFFECTS OF NUTRITION, REPRODUCTION, GENETICS, AND ENVIRONMENTAL FACTORS ON ANIMAL PRODUCTIVITY

Ruminal Fungi for Increasing Forage Intake and Animal Productivity

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ABSTRACT

Increased fungal activity in the rumen has the potential to increase production from ruminants by increasing dietary intake. To obtain new information on ruminal fungi involved in the breakdown of fibrous plant tissues, *Neocallimastix frontalis* was incubated with fragments of wood. Examination by electron microscopy has provided evidence for extra-cellular structures released by the fungus during degradation. The branched-linear structures, about 0.2–0.4 μm in length, migrated to and appear to attach by one end before aligning to form a thick layer on plant cell walls. We suggest that the structures migrating from *N. frontalis* contain multi-enzyme assemblages of polysaccharidases and are the fungal equivalent of cellulosomes released by many fibrolytic bacteria. When *Caecomyces communis* was incubated with plant tissue, the tissues were macerated and fibrillated as the fungus grew along fibres. This physical disruption was caused by bulbous rhizoids expanding inside the fragments. The novel breakdown process involved both rhizoidal contact with cell walls and degradation which occurred when small extracellular particles attached to cell walls. At some degradation sites, extracellular vesicles were also observed. The structure of the assemblages exported by *C. communis* differed markedly from those of *N. frontalis* probably reflecting the different modes of action for the two fungi. To locate activity from *Caecomyces* spp. in the rumen, fragments of ruminal digesta were examined. *Caecomyces* spp. were observed but only on the very small fragments indicating that these fungi are involved in the final stage of particle breakdown and fibre clearance from the rumen. Because of their specific fibre degrading properties it is suggested that this fungal genus is a key target for increasing feed intake and productivity in ruminants.

Key words: *fungi, rumen, forage intake, fibre breakdown and clearance, enzymes, physical disruption.*

INTRODUCTION

In grazing ruminants productivity is usually limited by the amount of feed an animal is able to ingest (intake). Because forage is the primary source of energy, the greater the intake the greater the production. However, intake is often restricted by slow clearance of resistant plant material from the rumen. Recalcitrant tissues are digested only slowly and thus inhibit further intake until they are released from the rumen as particles 1 to 2 mm in length (Ulyatt et al., 1986).

In the rumen, fibre breakdown and fibre clearance arise from a combination of two separate processes: physical breakdown of plant tissues during chewing and rumination, and fibre degradation during digestion. The digestion process is carried out by fibrolytic bacteria such as *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes*, fungi and some protozoa (Demeyer, 1981; Chesson and Forsberg, 1997). Of these microbes, the fungi are most promising targets for improving fibre breakdown (Orpin and Joblin, 1997). Ruminal fungi have been associated with increased feed intake in sheep (Gordon and Phillips, 1993) and with improved growth rates in buffalo calves (Tripathi et al., 2007).

This paper describes results from studies on two species of fungi; one which digests very recalcitrant lignocellulosic material, and one which has a novel capacity to physically disrupt and macerate fibrous plant tissues. Ruminal fungi are known to secrete a wide range of cell wall degrading enzymes (Joblin et al., 1990; Teunissen and Op den Camp, 1993) and the genes coding for some of these polysaccharidases have been cloned (Bassam et al., 1995 and refs therein). We present here results from a scanning electron microscopy (SEM) and transmission electron microscopy (TEM) examination of fungus/cell wall interactions inside plant fragments during degradation. The aim was to compare the modes of action of *Neocallimastix frontalis* and *Caecomyces communis*, species with different morphologies.

Neocallimastix frontalis has thin filamentous rhizoids (rhizomycelia) (Orpin, 1994), degrades cell walls in forage (Akin, 1994), and has been shown to digest resistant lignocellulosic tissue in some woods (Joblin and Naylor, 1989). In the present work, *Populus tremuloides* wood, readily degraded by *N. frontalis* (Joblin and Naylor, 1989) but not by the highly cellulolytic bacterium *R. albus* (Joblin and Naylor, unpublished), was selected as substrate because it provides thick secondary cell walls ideal for TEM examination.

In contrast to *N. frontalis*, fungi belonging to the *Caecomyces* genus, a reclassification of the *Sphaeromonas* genus (Gold et al., 1988), have non-filamentous rhizoids which are large and bulbous (Orpin, 1994). These appear ill-suited to penetrate plant cell walls but one study has shown that *C. communis* fibrillated plant tissue during fermentation (Joblin, 1989). In the present work, *C. communis* was incubated with sisal (*Agave sisalona* L) to examine interactions between the fungus and plant cell walls. After confirming the physical disruption of tissue, the study was extended to fragments from ruminal digesta. This showed that the mode of action of *Caecomyces* spp. in the rumen is similar to that observed *in vitro*.

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MATERIALS AND METHODS

Media and Cultures

The anaerobic culture techniques and methods for preparing media under O_2-free CO_2 were those used previously for culturing ruminal fungi on solid substates (Joblin, 1981; Joblin et al., 2002). All cultures were in screw-top Hungate tubes (Bellco Biotechnology, Vineland, NJ) and incubations were carried out at 39 °C. Medium B, a modification of the rumen fluid-containing medium of Joblin et al. (2002), consisted of per L: salts solution A (170 mL), salts solution B (170 mL), clarified ruminal fluid (300 mL), yeast extract (500 mg), trypticase (1 g), $NaHCO_3$ (5 g), distilled water (360 mL), 0.01% resazurin (0.3 mL), and cysteine hydrochloride (500 mg). For sisal medium, pieces of sisal twine (5–8 mm long) were added to each tube before addition of pre-reduced medium (10 mL).

A similar medium was prepared for *Populus tremuloides* (PT). Pieces of PT (approximately 12 mm × 5 mm × 3 mm) were removed from a block of wood using a razor blade, dried *in vacuo* over P_2O_5 for 24 h and 20–30 mg added to each tube prior to addition of medium B (10 mL). Media containing solid substrates were autoclaved at 121 °C for 15 min. For agar roll tubes, melted agar (at 41 °C) containing sisal fibres (3–5 mm long) was inoculated with *C. communis* zoospores and the tubes rolled at 12 °C to solidify agar. To provide zoospores for inocula, *N. frontalis* PNK2 and *C. communis* CS123 obtained from the Rumen Culture Collection now at the Grasslands Research Institute were grown for 3 d in sisal medium.

Microscopy

After incubation, culture fluid was removed by aspiration and fixative solutions added directly to culture tubes. For histochemistry, sections (0.5 µm) were cut with a diamond knife, stained with (0.05%) toluidine blue in 0.1M sodium phosphate buffer (pH 7.2) and examined by bright-field microscopy. For SEM, solid substrates were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 for 5 h at room temperature and then washed successively with the buffer for 5, 10 and 15 min. Substrate samples were post-fixed with 1% OsO_4 in the same buffer for 2 h at room temperature, washed twice with buffer, dehydrated and dried to critical point with liquid CO_2. For fixation of rumen contents, 8 mL of 25% glutaraldehyde was added to 200 g freshly collected digesta from a sheep fed fresh forage, mixed thoroughly and left for 24 h. For SEM of plant fragments from the rumen, samples were prepared as above. Specimens were mounted on Al stubs, sputter-coated with gold and examined using a Cambridge Model 250 Mk III Scanning Electron Microscope (Cambridge Instruments, UK). For TEM, samples fixed as before, were washed with 0.1 M potassium phosphate buffer (pH 7.2), post-fixed with OsO_4 at 4 °C, dehydrated and embedded in epoxy resin. Thin sections, cut with a diamond knife, were stained with uranyl acetate and co-stained with lead citrate before examination using a Philips 201C Transmission Electron Microscope.

RESULTS AND DISCUSSION

Neocallimastix frontalis

Chips of *P. tremuloides* wood were not degraded by *C. communis* but 31% (dry weight) was solubilised by *N. frontalis* after 6 d incubation (data not shown).

This result agrees with that of a previous study which showed that *P. tremuloides* was readily digested by *N. frontalis* and that cellulose, xylan and galactomannan were the major cell wall components degraded (Joblin and Naylor, 1989). *N. frontalis* releases highly active extracellular cell wall-degrading polysaccharidases (Wood et al., 1986; Joblin et al., 1990; Teunissen and Op den Camp, 1993). A light microscopy examination of chip sections (**Figure 1a**) revealed that the secondary cell walls were highly degraded whereas middle lamellae between cells were resistant to degradation. Cell wall degradation progressed successively from outer cells to inner cells with cell walls in the centre of chips showing little degradation (**Figure 1a**). The transfer of fungus between cells probably involved cell degradation of the type seen in forage cell walls (Akin, 1994) together with zoospore migration through pit apertures. Mature sporangia (fruiting bodies) containing zoospores were observed in inner cells (not shown). A fungal rhizoid penetrating a secondary cell wall is shown in **Figure 1d**.

In general, during degradation rhizoids grew in the lumen of cells rather than attached to cell walls (**Figures 1a–c**) and cell walls were degraded by erosion processes (**Figures 1b–c**) as would be expected for extracellular enzymes. This is in agreement with findings that the cell walls of forage are eroded by filamentous ruminal fungi during digestion (Akin, 1994).

Access to degraded *P. tremuloides* chips (**Figure 1a**) enabled us to select cell walls at differing stages of degradation for further examination. **Figure 2a** shows rhizoids together with an actively degrading secondary cell wall. The rhizoids have internal membraneous or hydrogenosome-like structures (Munn, 1994) and the degrading cell wall is covered with an electron-dense layer. At higher magnification (**Figure 2b**), numerous branched-linear structures (about 0.2–0.4 µm long) were observed between rhizoids and the degrading plant cell wall. These structures appear to be migrating towards the cell wall for attachment as expected for extracellular polysaccharidases (Chesson and Forsberg, 1997). The structures attach by one end and a layer is formed on the cell wall surface (**Figure 2b**). The appearance of the layer (**Figure 2b**) suggests that the structures are orientated parallel to rather than perpendicular to the cell wall during degradation.

Figure 2c shows a longitudinal section of a rhizoid in contact with an actively degrading cell wall. Examination at higher magnification (**Figure 2d**) revealed that the rhizoid is rich in polysomes, suggesting high polypeptide synthesis activity. The branched-linear structures present between rhizoid and plant cell wall and on the outer surface of the rhizoid (**Figure 2d**) have a similar appearance to the extracellular structures in **Figure 2b**.

To the best of our knowledge these extracellular structures have not been observed before during cell wall degradation by ruminal fungi. We suggest that they contain the multiple enzyme complexes necessary for cell wall degradation and are functionally equivalent to the cellulosomes released by many anaerobic bacteria during cell wall degradation (Bayer et al., 1998). This is supported by observations that one of the most active cellulases known is produced by *N. frontalis* (Wood et al., 1986) as part of a multi-enzyme complex similar to that found in cellulosomes (Wilson and Wood, 1992). The bacterial cellulosome contains at least 26 different polypeptides including structural proteins (Bayer et al., 1998). Genes encoding cellulosome-like components have been isolated from ruminal fungi (Nagy et al. 2007; Steenbakkers et al., 2008) and these include genes for both polysaccharidases and polypeptides with putative structural properties (e.g. dockerins) of the type found in cellulosomes (Raghothama et al., 2001; Steenbakkers et al., 2001 and refs therein).

Close examination of a region of cell wall which had been extensively degraded (**Figures 2e–f**) revealed that the electron-dense layer on the residual cell wall had a different appearance to that on actively-degrading cell walls (**Figures 2b, 2d**). In this case, the components of the layer appear to align perpendicular to the cell wall

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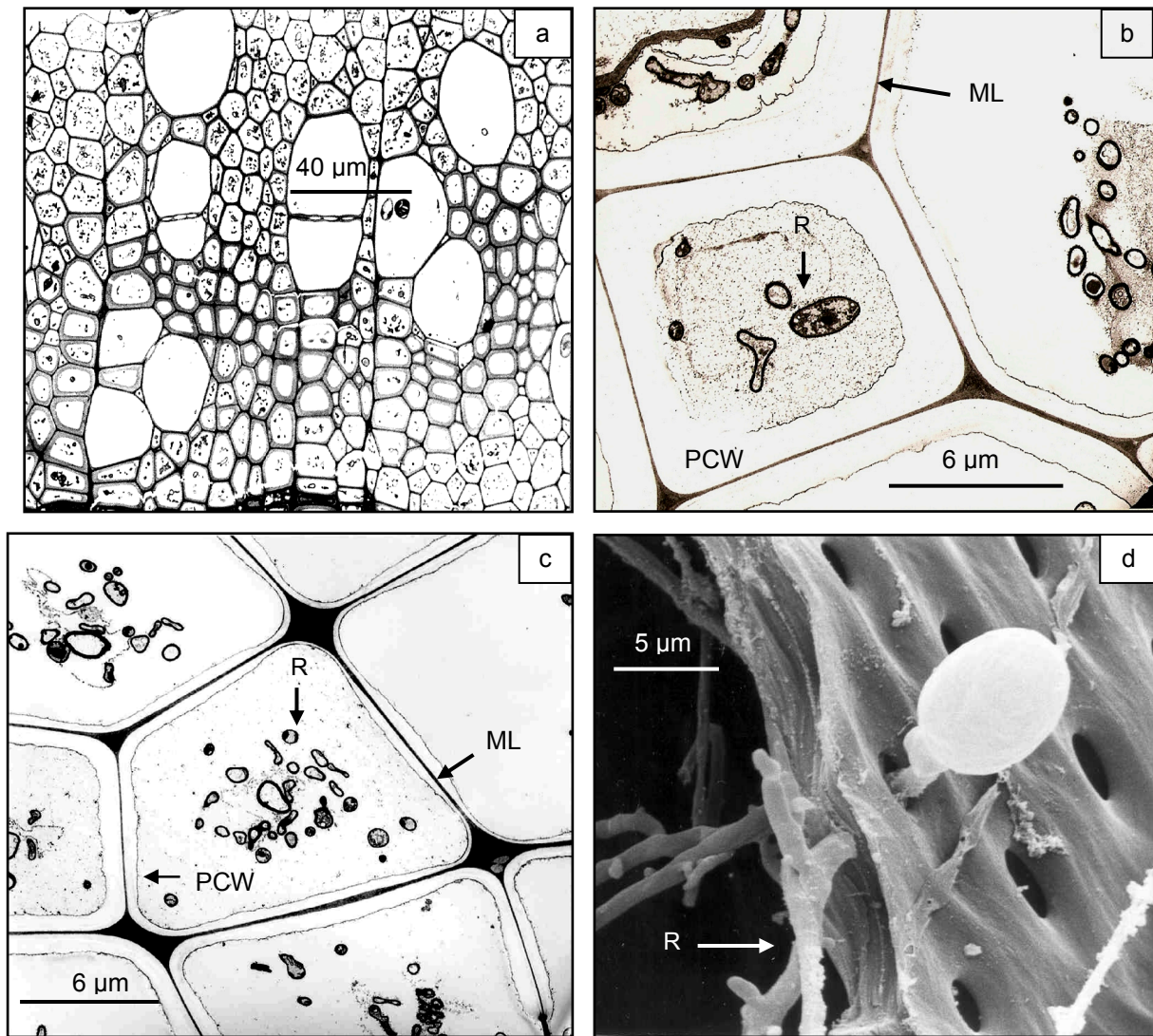
Figure 1. Degradation of *Populus tremuloides* wood by *Neocallimastix frontalis*. a — light micrograph of a chip section after 6 d incubation; b — TEM micrograph of actively degrading cell walls; c — TEM micrograph of extensively degraded cell walls; d — SEM micrograph of a rhizoid penetrating a pit-aperture cell-wall; R — rhizoids; ML — middle lamella; PCW — plant cell-wall.

(**Figure 2f**), perhaps as a consequence of interactions with lignin or polyphenolic components in the nearby middle lamella.

Caecomyces communis

Figure 3a shows sisal fibres in agar roll tubes after 6 d incubation. Fungal establishment began at the end of fibres (not shown). As the fungus grew and bulbous rhizoids expanded within fibres (**Figures 3c–d**) plant tissue became macerated and fibrillated (**Figures 3a–b**). Fracture planes developed between fibrils during fungal growth (arrows in **Figure 3c**) and at the completion of incubation tissues had a shredded appearance.

Our findings indicate that the disruption of plant tissues by *C. communis* arises predominantly from expansion of bulbous rhizoids within tissues and support previous observations (Joblin, 1989). Large bulbous rhizoids reached diameters of around 50 µm (**Figure 3b**) and contained little of the electron-dense material observed in small rhizoids (**Figures 3e–f**). The large bulbous rhizoids (**Figures 3c–3f**) probably contain mainly vacuoles (Wubah et al.,

1991). Examination by TEM (**Figures 3e–f**) showed that the process involved both contact of fungus with plant cell walls as well as cell wall degradation. Contact points (or attachments) between fungus and plant cell-wall are arrowed in **Figures 3e–f** and **Figures 4a–b**.

There was no degradation of middle lamellae (**Figures 3e–f**) but at sites of sisal cell wall degradation (**Figure 4**) small electron-dense particles appear to be migrating from fungal rhizoids to the plant cell-walls (**Figures 4b–d**) as would be expected for degradation involving extra-cellular enzymes. Extra-cellular cellulases and xylanases are known to be released from *C. communis* (Hodrová et al., 1998; Matsui and Ban-Tokuda, 2008). The particles formed electron-dense layers on degrading cell walls. At some degradation sites, extracellular vesicles were observed together with particles (**Figure 4c**). We suggest that the particles are the *C. communis* equivalent of bacterial cellulosomes. It is noteworthy that these putative multi-enzyme assemblages differ markedly in structure from those released from *N. frontalis* (**Figures 2b, 2d**) suggesting that the macromolecular structure of the polysaccharidase-complexes released from *C. communis* is very different to those of *N. frontalis*.
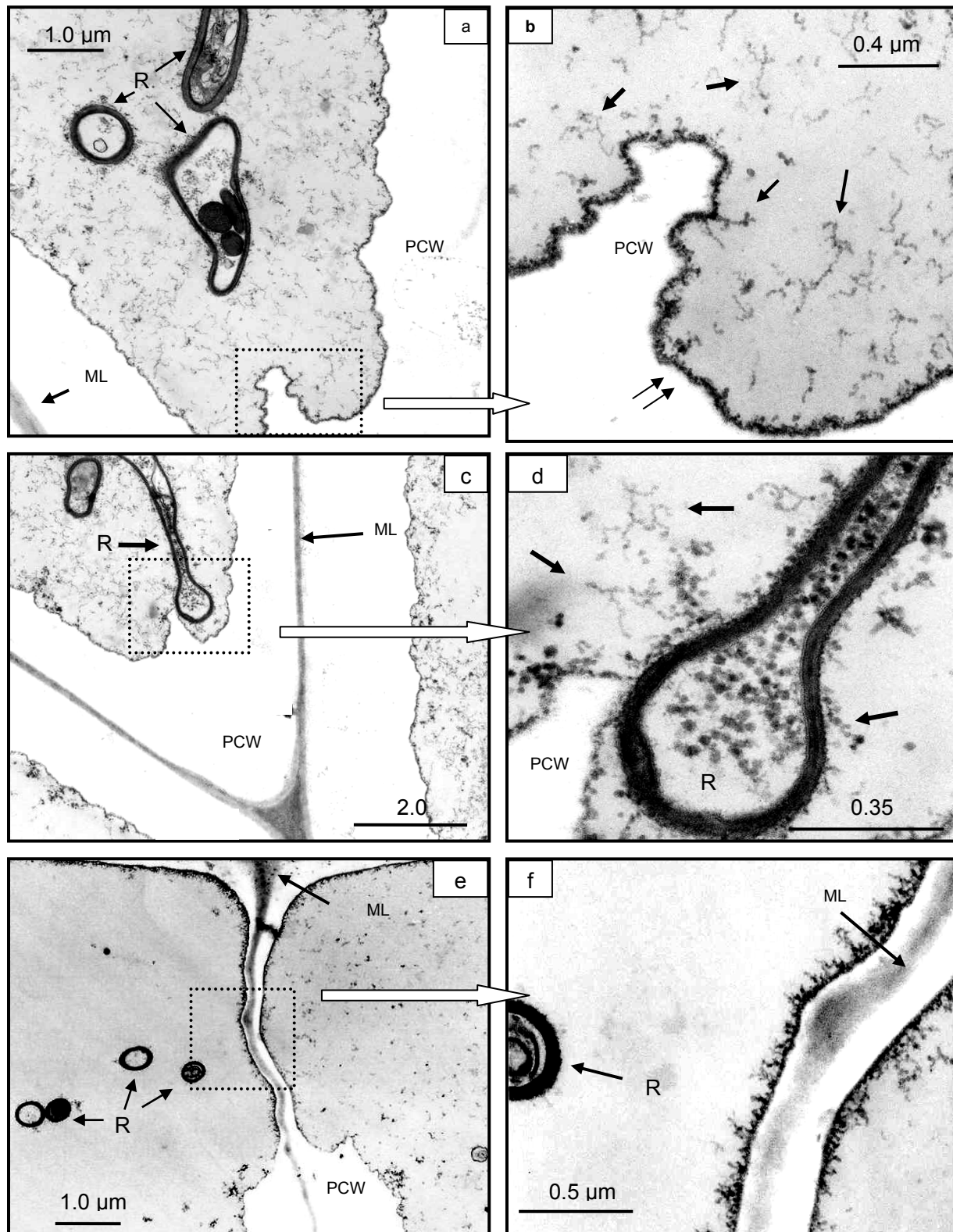
Figure 2. Degradation of *Populus tremuloides* cell-walls by *Neocallimastix frontalis.* TEM micrographs showing: a and b — rhizoids, extracellular structures (arrows) and the electron-dense layer (double arrows) on a degrading cell-wall; c and d — rhizoid in contact with degrading cell wall and extracellular structures (arrows); e and f — extensively degraded cell wall with attached layer components perpendicular to the cell wall. PCW — plant cell-wall; ML — middle lamella; R — rhizoid.
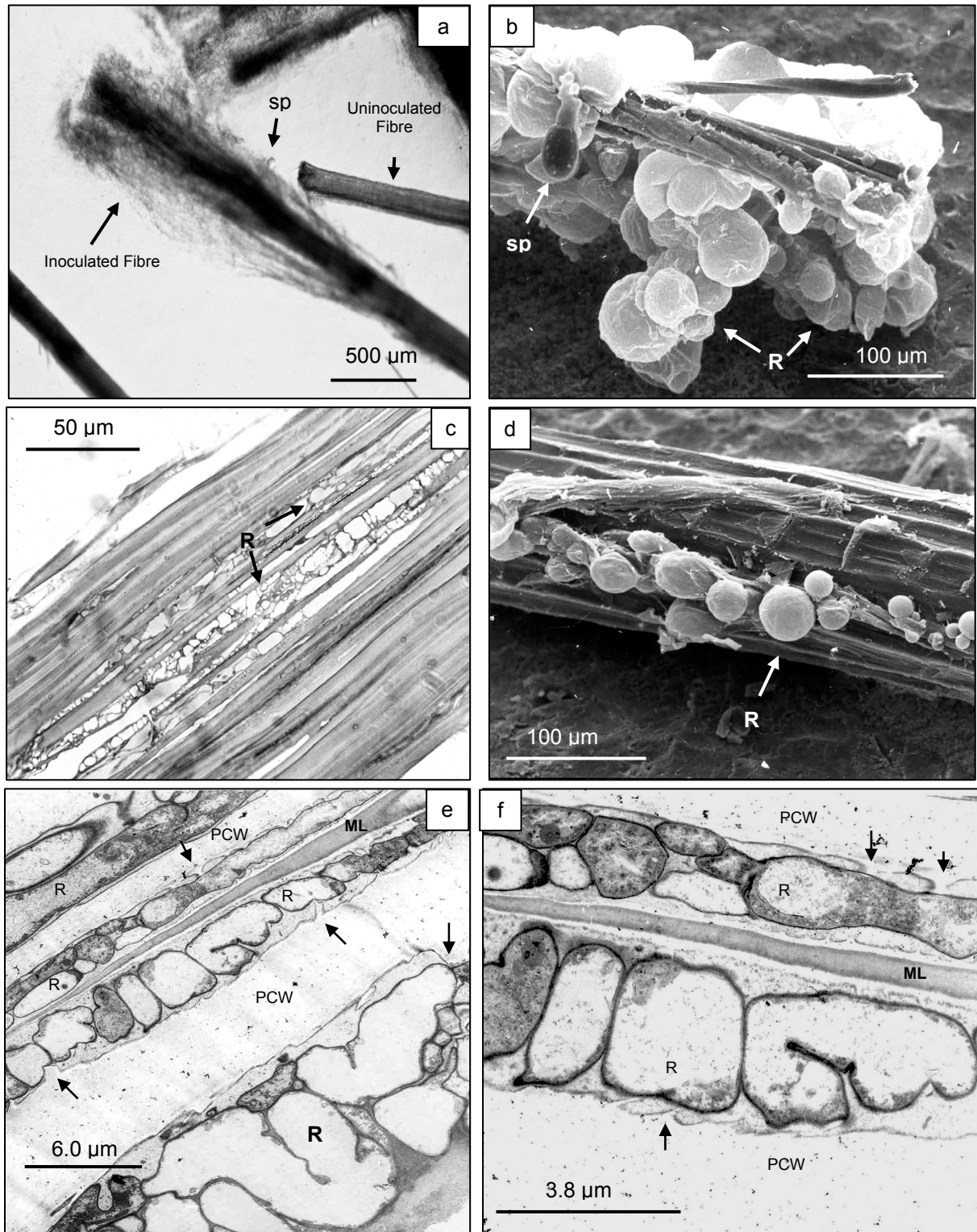
Figure 3. Breakdown of sisal fibre by *Caecomyces communis*. a — light micrograph showing fibre disruption; b — SEM micrograph of fibrillated tissue and rhizoids; c — light micrograph showing internal growth of rhizoids; d — SEM micrograph showing fibre fracture; e and f — TEM micrographs showing internal rhizoid growth and rhizoid/cell-wall contacts (arrows). SP — sporangium; PCW — plant cell wall; ML — middle lamella; R — rhizoids.
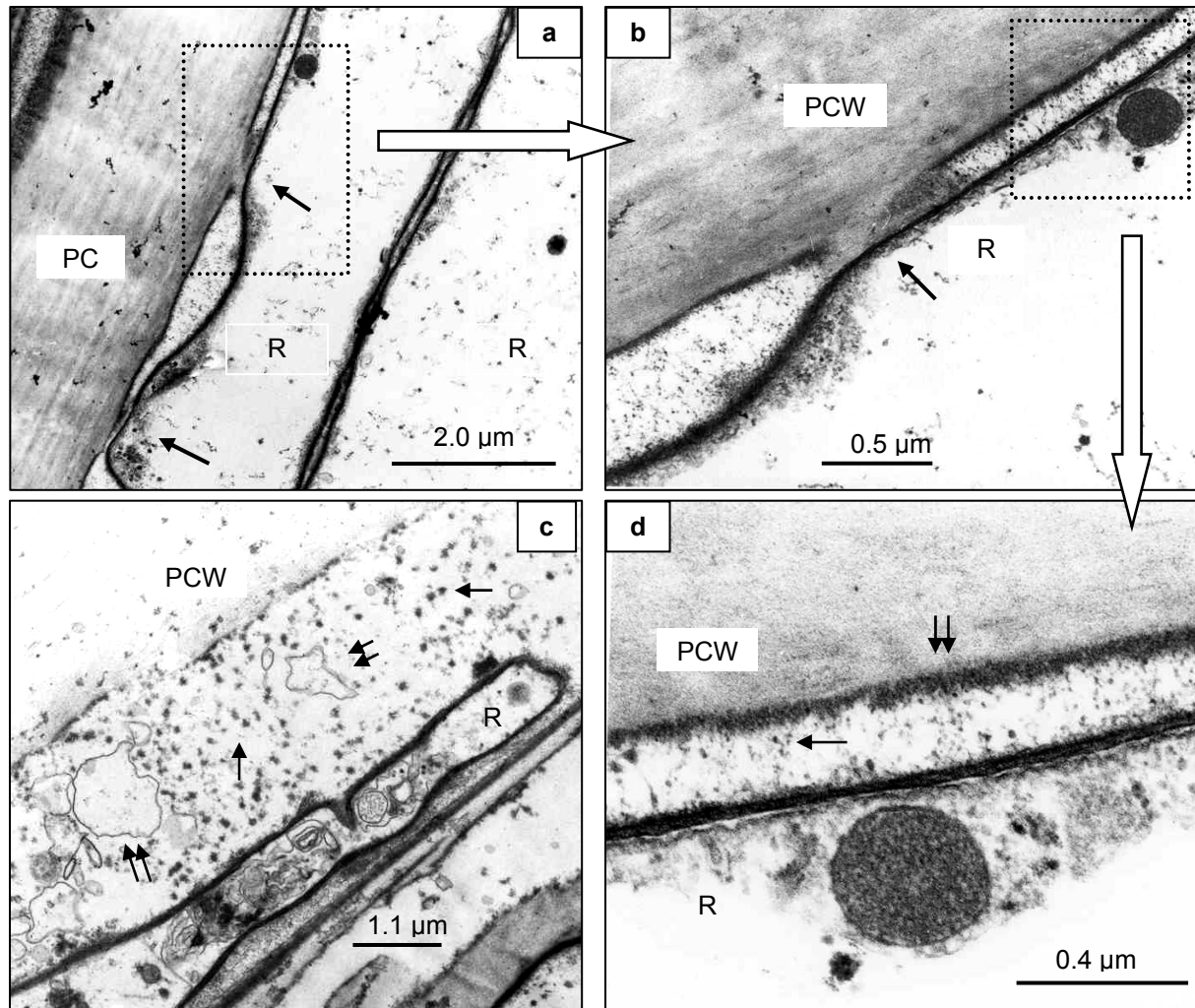
Figure 4. TEM micrographs of sisal cell wall degradation by *Caecomyces communis*. a and b — rhizoid contact with cell wall (arrows); c — extracellular particles (arrowed) and vesicles (double arrows) between rhizoid and degrading cell wall; d — particles (arrows) between rhizoid and cell wall and electron-dense layer (double arrows) on cell wall. PCW — plant cell wall; R — rhizoid.

To find evidence of *Caecomyces* spp. activity *in vivo*, plant fragments from a sheep rumen were investigated. An SEM examination found sporangia similar to those of *N. frontalis* (Orpin and Joblin, 1989) on fragments but, despite extensive efforts, failed to find caecomyces-like rhizoids. In a final study, small fibrillated fragments which had been discarded were examined. This revealed the presence of bulbous rhizoids (**Figure 5**) on many particles. The rhizoids had a similar appearance to those of *C. communis* growing *in vitro* (**Figures 3b, 3d**) and grew parallel to the axis of plant fibres (**Figures 5a–c**) as observed in sisal fragments incubated with *C. communis* (**Figure 3d**). These findings show that *Caecomyces* spp. are involved in the breakdown of small particles in the rumen. We suggest that this is their ecological role. It is likely to be a key process in fibre clearance because only the small particles are released from the rumen during digestion (Ulyatt et al., 1986).

CONCLUSIONS

The rumen is essentially a fermentation vat of microbes adapted to digesting lignocellulosic tissues in order for the host animal to thrive on a forage diet. Ruminant production would be revolutionised if fermentation in the rumen could be controlled in the manner of industrial fermentations which produce antibiotics, metabolites, enzymes, microbial cells etc. A major goal is to increase fibre clearance.

This study has shown that the fibre breakdown abilities of *Caecomyces* spp. are unusual. They fragmented and macerated plant tissues in a manner leading directly to particle size reduction — a property not found in other fungi. In the rumen they appear to have a specific role in fragmenting small particles such as those released during chewing and rumination — and which require further size reduction before release from the rumen. When large stem-cylinders from alfalfa hay were incubated with *C. communis* (data not shown), the waxy cuticles were removed intact. Removal of the protective cuticle would expose fragment surfaces to increased attack from ruminal bacteria. This 'cuticle peeling' property requires further investigation because it was not always reproducible in *in vitro* incubations. It is concluded that these fungi play a key role in fibre clearance from the rumen. Inoculation of animals with more effective and competitive *Caecomyces* strains, or methods which enhance
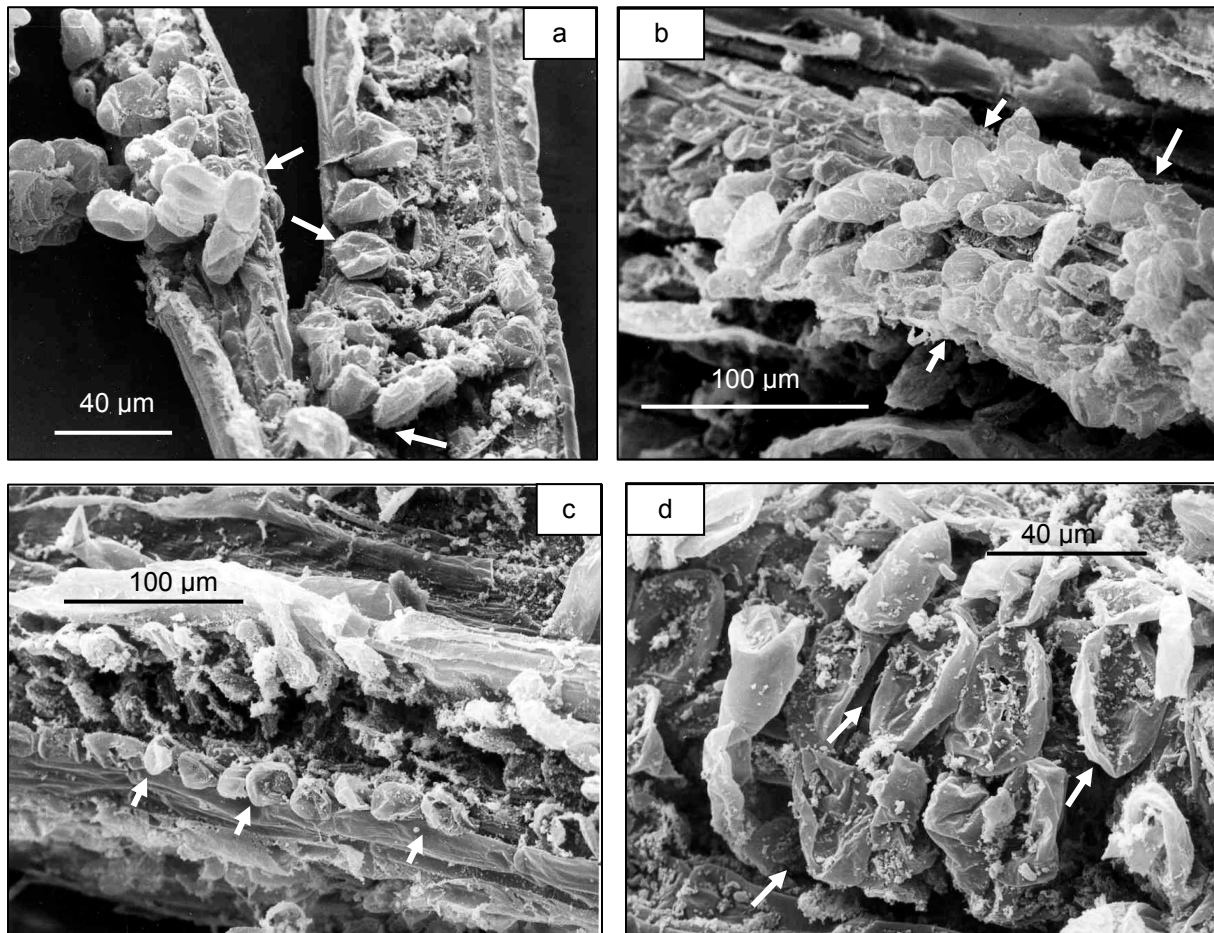
Figure 5. SEM micrographs showing rhizoids (arrows) of *Caecomyces* spp. on small macerated particles in ruminal digesta collected from a sheep fed forage.

Caecomyces spp. activity *in vivo* are likely to lead to increased intake and thus productivity.

This study also provides the first observations on structures moving from ruminal fungi to plant cell walls. The behaviour of the structures is consistent with their containing enzymes responsible for cell wall degradation so we conclude that they are multi-enzyme assemblages functionally equivalent to bacterial cellulosomes. Access to these should now allow their properties to be determined. This will provide new information for biotechnologies aimed at improving rumen function. We recognise that our evidence is indirect and experimental proof for the 'cellulosomes' is required. Unfortunately, further studies such as an immuno-cytochemical investigation using labelled monoclonal antibodies targeted at cellulosome components (cellulases, xylan-esterases, dockerins etc) were beyond the scope of our work.

ACKNOWLEDGEMENTS

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Effect of Ethyl Linolenate on Rumen Fermentation and Microbial Community in Sheep Fed Diets with Different Forage to Concentrate Ratios

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ABSTRACT

A two-way factorial arrangement was conducted to investigate the effect of ethyl linolenate (LNE) on rumen fermentation and microbial community in sheep fed diets with different forage to concentrate ratios (F:C). Four male Hu sheep were fistulated and each was paired with a non-fistulated animal, and then the four pairs of animals were fed a forage-based or a concentrate-based diet without or with LNE. Addition of LNE decreased methane (CH_4) emission by 17.3 and 33.8% in forage- and concentrate-based diets respectively, with a significant interaction between the diet and LNE ($P < 0.05$). Total volatile fatty acids were little affected by the diet, but decreased in the LNE-added group ($P < 0.05$). Addition of LNE decreased the molar proportions of acetate and butyrate, and increased the molar proportion of propionate in concentrate-based diet ($P < 0.05$), but this was not the case with the forage-based diet. Microbial protein mass was decreased significantly by inclusion of LNE ($P < 0.05$). Reducing the F:C ratio significantly decreased the population of fungi and *R. albus*, but had a minor effect on methanogen protozoa, *R. flavefaciens* and *F. succnogen*. Addition of LNE significantly decreased the population of methanogen and protozoa, but had a minor effect on fungi and *F. succnogen*. It is inferred that interactions of fat with the basal diet have to be taken into consideration when developing effective CH_4-abatement feeding strategies.

Key words: *ethyl linolenate, sheep, fistulated, rumen, volatile fatty acids, microbial protein, methane*

INTRODUCTION

Methane is produced as an unavoidable by-product of organic matter fermentation in the rumen and represents a two to 12% loss of gross energy intake (Johnson and Johnson, 1995). The concentration of CH_4 in the atmosphere has increased at a rate of 10 nL/L per year since the preindustrial revolution (Moss et al., 2000). Domesticated ruminants are estimated to produce about 80 Tg of CH_4 annually (1

Tg = 1 million metric tons), accounting for about 22% of CH_4 emissions from human-related activities (NRC, 2002). Therefore, reducing CH_4 emission from ruminants has implications not only for global environmental protection but also for efficient animal production.

Many potential strategies have been suggested to reduce CH_4 production (Moss et al., 2000). However, many of these options are at an early stage of development, or in the case of ionophores, are proscribed by European legislation. For increasing animal productivity and thereby reducing CH_4 production per unit of animal product, the main avenue available is the alteration of ruminal fermentation patterns through dietary manipulation, primarily the substitution of structural with non-structural carbohydrates and the dietary inclusion of fatty acids (Moss et al., 2000) which are normally added to increase energy density, enhance milk production, or modify the fatty acid composition of milk (Zheng et al., 2005; Sanz Sampelayo et al., 2007). In a previous study with different types and levels of octadecacarbon fatty acids, it was found that linolenic acid had the most efficient CH_4-suppressing effect *in vitro* (Zhang et al., 2008). However, there are few studies on dietary interactions with linolenic acid. In this trial, the effect of ethyl linolenate (LNE) on CH_4 emission and rumen fermentation was investigated in sheep given diets differing in the forage to concentrate ratio (F:C) using simple open-circuit respiratory chambers.

MATERIALS AND METHODS

Experimental Design, Animals and Feeds

The experimental design was a 4 × 4 Latin square with 2 × 2 factorial arrangement of four dietary treatments. Four male Hu sheep were fistulated and each was paired with a non-fistulated animal at the beginning of the experiment and the pairing of animals was maintained throughout the trial. Four pairs of sheep were fed a forage-based diet without (F; F:C = 70:30, dry matter [DM] basis) or with LNE (FL; F:C = 70:25, 5% LNE); a concentrate-based diet without (C; F:C = 30:70) or with LNE (CL; F:C = 25:70, five percent LNE), respectively. The LNE was purchased from Henan Linuo Biochem Co., Ltd., China and its α-linolenic acid content is above 70%. The LNE was poured onto the feed and mixed into the ration manually at the time of feeding. The feed amount of the experimental diets was adjusted to the live weight at the start of experiment and was kept constant afterwards. Each sheep was fed one kg of total feed (DM) including concentrate and alfalfa hay/d and were consumed without refusals. Diet ingredient and chemical composition are shown in

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Table 1. Ingredients and chemical composition of diets fed to sheep.

Diet[1]	Forage-based		Concentrate-based	
Item	F	FL	C	CL
Ingredient, g/kg				
Alfalfa hay	700	700	300	300
Ethyl linolenate	0	50	0	50
Corn	152	90	450	388
Soybean meal	78	90	100	112
Rapeseed cake	30	30	50	50
Wheat bran	26	26	86	86
Salt	4	4	4	4
Vitamin and mineral premix	10	10	10	10
Chemical composition, g/kg DM				
Crude protein	153.1	153.0	153.2	153.0
Neutral detergent fibre	394.4	390.2	253.9	249.8
Acid detergent fibre	22.8	22.3	45.9	45.4
Calcium	9.2	9.1	4.5	4.6
Phosphorus	3.2	3.2	2.3	2.2
DE, MJ/kg DM	93.3	102.0	118.0	126.7

[1]F — a forage-based diet without LNE; FL — a forage-based diet with 50 g/kg LNE; C — a concentrate-based diet without LNE; CL — a concentrate-based diet with 50 g/kg LNE.

Table 1. The diets were given in equal portions twice daily at 0800 h and 1600 h. During the whole experiment, the sheep had free access to fresh water.

Sampling Procedures and Measurements

Methane Measurement
Each period lasted for 25 d. During the first 22 d of each period, each pair of sheep was housed, untethered in individual pens. The pens were located in a sheltered, unheated barn. Before the morning feeding on d 23, the first two pairs of sheep were moved to one of chambers for measurements of CH_4. Because only two chambers were available, two pairs of animals were used at the same time. Within each chamber, the animals were untethered and had free access to fresh water. The first day within the chamber was considered an adjustment period, allowing the sheep to adapt before measurements were recorded for two consecutive 24-h d starting at 0800 h. After the morning measurements, the sheep were removed from the chambers and transported to their individual stalls. Then another two pairs of sheep were used for measurements. The gas sampling and monitoring techniques were as described previously (Yuan et al., 2007). Briefly, during the two consecutive d when the sheep were housed in chambers, air samples were taken hourly from each chamber with an airproof syringe, the volume of the air that flowed through the chamber was recorded, and CH_4 concentrations were analysed using a gas chromatograph (GC-2100, Shimadzu) equipped with a flame ionisation detector (FID) (Hu et al., 2005a).

Rumen Sampling
On the last d of each period, rumen fluid samples were taken from fistulated sheep before morning feeding using a vacuum bump. Immediately after collection, the samples were strained through four layers of compressed gauze and the pH was determined using a pH meter (Model PB-20, Sartorius). The fluid was sampled to determine ammonia-N, volatile fatty acids (VFA) and microbial protein (MCP) using the methods described by Hu (2005a). For determination of the relative quantity to total bacterial 16S rDNA of methanogens, protozoa, fungi, *R. flavefaciens R. albus* and *F. succinogenes*, six aliquots of one ml rumen fluid were sampled and stored immediately at -80 °C .

Total DNA Extraction and Real-time Quantitative PCR
Total DNA was extracted from rumen fluid by the bead-beating method as described by Zhang et al. (2008). The primers of total bacteria, methanogens, protozoa, fungi, *R. flavefaciens*, *R. albus* and *F. succinogenes* are as described by Denman and McSweeney (2006) and Denman et al. (2007). The species-specific real-time quantitative PCR was performed using the ABI 7500 real time PCR system (Applied Biosystems, USA) with fluorescence detection of SYBR green dye. Amplification conditions were as follows: one cycle at 95 °C for 10 s for initial denaturation, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplification efficiencies for each primer pairs were investigated by examining a dilution series of total rumen microbial DNA template on the same plate in triplicate.

Calculation and Statistical Analysis
Populations of rumen microbes were expressed as a proportion of total rumen bacterial 16S rDNA according to the equation: relative quantification of target $= 2^{-(Ct\ target\ -\ Ct\ total\ bacteria)}$, where Ct represents the threshold cycle.

All data were analysed as a four×four Latin square using the mixed procedure of SAS (1999). The statistical model included

sheep as random effect, period, LNE addition, F:C, and LNE×F:C as fixed effects. Main effects (F:C and LNE addition) and interactions between F:C ratio and LNE addition were considered to be significant when P ≤ 0.05.

RESULTS AND DISCUSSION

Effects on Methane Emission and Fermentation Characteristics

Addition of LNE decreased CH_4 emission by 17.3 and 33.8% in forage- and concentrate-based diets, respectively (**Table 2**). This reduction may be attributed to several interrelated factors. Total rumen hydrogen supply would be reduced primarily through a reduction in the total amount of ruminally fermented organic matter (Beauchemin and McGinn, 2006; Jordan et al., 2006), together with a shift in the ratio of the end products of fermentation from acetate towards propionate (Wettstein et al., 2000). Rumen hydrogen supply available for reducing CO_2 to CH_4 would be further reduced by the LNE serving as an alternative hydrogen sink through bio-hydrogenation, although the total amount of metabolic hydrogen used in the bio-hydrogenation of unsaturated fatty acids is small compared with that used for reducing CO_2 to CH_4 (Czerkawski, 1986). In addition, by reducing the rumen ciliate population, interspecies hydrogen transfer would be reduced and overall CH_4 production lowered (Finlay et al., 1994; Hu et al., 2005b).

Reducing the F:C ratio of diets led to a significant reduction in CH_4 emissions in the current study (P < 0.05, **Table 2**). Methanogenesis is an important terminal step in the anaerobic fermentation of organic matter within the rumen. Carbohydrates are the main energy source for the rumen microbes and the production of CH_4 is closely related to their fermentation. Compared with structural carbohydrates, the fermentation of non-structural carbohydrates (starch, sugars, etc.) results in less CH_4 per unit of substrate fermented (Hungate, 1966). Thus, increasing the dietary proportion of concentrate, i.e. the proportion of easily fermentable carbohydrates, appears to be an effective feeding strategy in decreasing rumen methanogenesis (Lovetta et al., 2003). A decline in rumen pH in the lower F:C diet (**Table 2**) might also contuibute to the reduced CH_4 production, since

rumen methanogenesis was shown to be a pH-dependent process (Van Kessel and Russell, 1996).

Highly significant interactions in CH_4 emission existed between the basal diets and LNE addition, with greater responses for diets with a lower F:C ratio (P < 0.05). This may be attributed to the different amount and structure of the dietary particulate matter between the two F:C diets (Machmüller, 2006). Harfoot (1974) demonstrated that fatty acids may attach either to rumen microbes or to feed particles. In the present study, the decrease in CH_4 emissions by LNE in the forage-based diet was about half of the decrease achieved with the concentrate-based diet. Since the two basal diets had little effect on average rumen pH, it can be assumed that this was mainly a result of the different amount and structure of the dietary particulate matter. With the forage-based diet, probably more LNE was attached to the feed particles and less LNE to the methanogens compared to the concentrate-based diet.

Diet type had no effect on total VFAs, but LNE addition decreased total VFAs significantly (P < 0.05, **Table 2**). Molar proportions of acetate and butyrate decreased, while molar proportions of propionate increased significantly by addition of LNE in concentrate-rich diet (P < 0.05), but they were not affected in forage-rich diet (P > 0.05). Thus, the acetate-to-propionate ratio was significantly decreased in the concentrate-rich diet. The LNE treatment affected rumen fermentation patterns in a manner similar to that shown previously by McGinn (2004) in a study in beef cattle, with a lower VFA concentration and a smaller acetate:propionate ratio. Ammonia-N concentration and MCP were reduced significantly by addition of LNE (P < 0.05), but diet type had little effect on MCP yield (**Table 2**). These results are consistant with most previous observations (Hristov et al., 2004).

Effects on Rumen Microbes

The influence of LNE and diet on ruminal microbial population is shown in **Table 3**. Methanogen and protozoan populations were decreased significantly (P < 0.05) by addition of LNE, but not affected by the F:C ratio or their interaction (P > 0.05). This indicates that LNE reduces CH_4 emission mainly by reducing the quantities of methanogens and protozoa quantity; this is in line with previous observations *in vitro* (Zhang et al., 2008). The low methanogen population relative

Table 2. Methane emission and ruminal parameters for sheep fed diets containing a forage-based diet without (F) or with 50 g/kg LNE (FL), and a concentrate-based diet without (C) or with 50 g/kg LNE (CL).

Diet	Forage-based		Concentrate-based		SEM	*P*-Value		
Item	F	FL	C	CL		F:C	LNE	Int
Methane (L/kg DM intake)	28.9[a]	23.9[b]	26.6[a]	17.6[c]	0.8	<0.01	<0.01	0.02
Ruminal pH	7.14[ab]	7.33[a]	6.90[b]	7.13[ab]	0.09	0.02	0.03	0.84
VFA (mmol/L)	68.6[a]	54.6[ab]	62.2[ab]	49.7[b]	4.1	0.26	0.02	0.88
Molar proportions (%)								
Acetate	77.4[a]	76.9[a]	71.7[b]	69.3[c]	0.7	<0.01	0.14	0.30
Propionate	13.7[b]	14.8[b]	16.6[b]	22.6[a]	1.3	<0.01	0.03	0.11
Butyrate	9.0[ab]	8.3[b]	11.7[a]	8.1[b]	0.8	0.15	0.02	0.10
A:P	5.74[a]	5.35[ab]	4.38[b]	3.16[c]	0.30	<0.01	0.08	0.34
NH_3-N (mg/dL)	12.3[c]	10.7[d]	20.7[a]	16.8[b]	0.4	<0.01	<0.01	0.15
MCP (mg/mL)	1.95[a]	1.55[b]	1.95[a]	1.69[ab]	0.11	0.25	0.01	0.76

[a, b, c, d] Means within the same row sharing no common capital letters are different at P < 0.05.

LNE — ethyl linolenate; Int — interaction between F:C and LNE; VFA — volatile fatty acids; A:P — acetate-to-propionate ratio; MCP — microbial crude protein.

Table 3. Ruminal microbes for sheep fed diets containing a forage-based diet without (F) or with 50 g/kg LNE (FL), and a concentrate-based diet without (C) or with 50 g/kg LNE (CL).

Diet	Forage-based		Concentrate-based		SEM	P-Value		
Item	F	FL	C	CL		F:C	LNE[a]	Int[b]
Methanogen	1.70[a]	0.67[b]	1.39[a]	0.43[b]	0.13	0.29	<0.01	0.88
Protozoa	2.84[a]	0.61[b]	2.80[a]	0.43[b]	0.25	0.57	<0.01	0.54
Fungi ($\times 10^{-4}$)	43.63[a]	15.28[b]	2.54[b]	0.89[b]	3.50	<0.01	0.07	0.10
R. flavefaciens ($\times 10^{-4}$)	2.79[b]	14.97[a]	3.34[b]	10.38[ab]	1.37	0.58	0.02	0.48
R. albus ($\times 10^{-2}$)	1.33[ab]	5.45[a]	0.13[b]	1.39[ab]	0.94	0.03	0.03	0.20
F. succnogen ($\times 10^{-2}$)	7.224[a]	4.034[ab]	3.516[ab]	0.004[b]	1.200	0.19	0.25	0.95

[a, b] Means within the same row sharing no common capital letters are different at P < 0.05.

LNE — ethyl linolenate; Int — interaction between F:C and LNE.

to total bacterial 16S rDNA associated with adding LNE may also be due to reduced hydrogen availability in the rumen. Methanogens live by consuming hydrogen in the rumen and have to compete with propionate-producing microbes that also consume hydrogen to form propionate. An increase in the molar proportion of propionate with LNE addition (**Table 2**) led to lower availability of hydrogen for methanogens.

Reducing the F:C ratio decreased the population of fungi and *R. albus* significantly (P < 0.05), but had minor effects on *R. flavefaciens* and *F.succnogen* (**Table 3**). Addition of LNE significantly decreased fungi number (P < 0.05), but promoted *R. flavefaciens* and *R. albus* populations (P < 0.05), with little effect on *F.succinogenes* (P < 0.05). No significant interactions between the F:C ratio and LNE addition were observed on populations of all the microbes (P < 0.05). Anaerobic fungi in the rumen mainly display strong cellulase and xylanase activity, and some of their plant cell wall degrading activities are through the physical action of rhizoid development, resulting in disruption of feed structure (Akin et al., 1989). Thus, fungi populations decreased significantly because of the markedly decreased structural carbohydrates with reducing F:C ratios. The different responses of fibrolytic microbes to LNE may be attributed to competition between them. Competitive and cooperative interactions between cellulolytic microorganisms may affect the degradation of fibrous feed and hence the energy provided to animals. Different substrates may affect the competitive status. Odenyo et al. (1994) observed that *R. flavefaciens* FD-1 competed with *F. succinogenes* S85 when cellulose was used as the carbon source, while the relative proportions of the two bacteria were similar until the substrate was depleted in alkaline hydrogen peroxide-treated wheat straw culture. In the study by Chen et al. (2008), sodium hydroxide treated rice straw increased liquid-associated *R. flavefaciens* and *R. albus*, but decreased *F. succinogenes* markedly in a mixed culture.

CONCLUSIONS

The feeding of low F:C ratio diets to Hu sheep is an effective way to reduce CH_4 emissions. By adding LNE to the diet, further reductions in CH_4 emissions can be achieved. There was a significant interaction between the basal diet and LNE addition in CH_4 production, with greater responses for diets with a lower F:C ratio. Addition of LNE significantly decreased populations of methanogen, protozoa and *F. succinogenes*, but promoted populations of *R. flavefaciens* and *R. albus*. Diet type had a significant effect on fungal growth, with minor effects on other microbes. Competition may exist among different fibrolytic bateria, resulting in greater populations of *R. flavefaciens* and *R. albus* in the LNA added diet than in the control. Interactions of

fat with the basal diet should be taken into consideration to develop effective CH_4-abating feeding strategies.

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Effects of Essential Oil from *Cordia verbenacea* D.C. on In Vitro Rumen Fermentation

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ABSTRACT

The objective of this experiment was to determine the effects of *Cordia verbenacea* D.C. essential oil (EO) on ruminal fermentation by using the *in vitro* gas production technique. Two substrates were independently assessed: i) Coastcross (*Cynodon* sp.) hay, and ii) 80:20 concentrate:forage diet. Treatments were defined as: Control i.e. without monensin or EO; MON i.e. monensin at 3 mM as a positive control; COR37.5 i.e. 37.5 mL of EO in 75 mL of buffered rumen fluid; and COR75 i.e. 75 mL of EO in 75 mL of buffered rumen fluid. Considering both substrates, MON reduced gas and methane (CH_4) production, increased propionate concentration, and decreased acetate:propionate ratio when compared with the Control. The most promising effect observed with EO inclusion was related to the inhibition of methanogenesis using hay as substrate. Methane produced per unit of $OM_{incubated}$ was reduced by 30% when COR75 was compared with Control. Although not statistically different, CH_4 production expressed as mL/g $OM_{degraded}$ showed an intermediary value for COR75 (32.9) compared with the Control (38.9) and MON (25.8). No effects were observed with EO inclusion when the high concentrate diet was used as substrate. In this condition, the doses tested seemed too low to manipulate rumen fermentation. The results indicate that the EO from *Cordia verbenacea* D.C. was able to modify *in vitro* ruminal fermentation using hay as substrate and that doses greater than 1 mL/mL of buffered rumen fluid may decrease CH_4 production as much as monensin.

Key words: *gas production, methane, plant secondary compounds, rumen manipulation.*

INTRODUCTION

Ionophoric antibiotics are the most common commercial feed additives used to manipulate rumen fermentation and enhance feed efficiency (Russell and Strobel, 1989). However, based on public health concerns the European Union banned the use of antibiotics as animal growth promoters in 2006 (European Commission, 2003). Apart from the debate derived from this decision (Russell and Houlihan, 2003), other countries, especially world beef exporters, probably will be pressed to follow this legislation in the near future.

In an attempt to reproduce the benefits of ionophores (e.g. monensin sodium), research has been exploiting the antimicrobial properties of plant secondary metabolites (PSMs; Calsamiglia et al., 2007). Plant secondary metabolites have some advantages over antibiotics, mainly because they are well accepted by consumers and generally considered safe for human consumption by regulatory agencies. Moreover, the appearance of PSM-resistant microorganisms is very unlikely because PSMs are a complex mixture of active components possessing a broad mode of action (Acamovic and Brooker, 2005). Conversely, these characteristics also reduce the selectivity against specific microbial populations, which impairs rumen manipulation (Calsamiglia et al., 2007).

Cordia verbenacea D.C. (Boraginaceae) is a Brazilian bush which has antimicrobial properties attributed to its essential oil (EO). A previous report using the plate diffusion method showed that 89% of the gram-positive bacteria tested were sensitive to this EO, whereas 80% of gram-negative bacteria were resistant (Carvalho Jr. et al., 2004). Thus, the hypothesis behind the research described below is that the EO from *C. verbenacea* would modify rumen fermentation and could mimic the positive effects observed for ionophores on ruminal fermentation. The objective of the experiment was to determine the effects of EO from *C. verbenacea* on ruminal fermentation by using the *in vitro* gas production technique. Two substrates were independently assessed: Coastcross (*Cynodon* sp.) hay and a 80:20 concentrate:forage diet. Monensin (MON) at 3 mM was included as a positive control.

MATERIALS AND METHODS

Experimental Design

The study was conducted from June to September 2008 at the Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil. It used the *in vitro* gas production technique and adapted to a semi automatic system using a pressure transducer and a data logger. A complete randomised block design was used with six replicates for gas production variables (mL/g $DM_{incubated}$ and mL/g $OM_{incubated}$) and three replicates for all other variables. Two conditions were independently assessed: i) Coastcross (*Cynodon* sp.) hay as substrate + an inoculum from sheep on pastures, and ii) an 80:20 concentrate:forage diet as substrate + an inoculum from sheep adapted to this diet. Two different inocula (blocks) were used as source of variation for each incubation condition. Treatments were defined as Control: without MON or EO; MON: MON at 3 mM

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as a positive control; COR37.5: 37.5 µL of EO in 75 mL of buffered rumen fluid; and COR75: 75 µL of EO in 75 mL of buffered rumen fluid.

Incubation Conditions

Serum glass flasks (total volume 160 mL; head space 85 mL) were filled with 500 mg of air-dried substrate (Coastcross hay or high concentrate diet), 50 mL of incubation medium (Theodorou's medium described in Preston, 1995), and 25 mL of rumen fluid. Flasks without substrate served as blanks to correct for gas release (n = 4), CH_4 production (n = 2), and residual dry matter (DM) and organic matter (OM) (n = 2) originating from the inoculum. Flasks filled with a standard Coastcross hay (n = 4) were also included to monitor incubation conditions. The pre-warmed flasks containing substrate were filled sequentially with incubation medium, MON solution or EO, and inoculum. Flasks were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), swirled manually, and incubated in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) at 39°C. Incubation time was 24 h for the hay and 16 h for the high concentrate diet (Makkar, 2004). Gas pressure was recorded at 4, 8, 12, and 16 or 24 h. For CH_4 analysis, 2.5 mL gas were sampled at each incubation time using a 5 mL syringe (Becton Dickson, Indústria Cirúrgica LTDA, Curitiba, PR, Brazil) and stored in a 10-mL vacuum tube. After each gas sampling, flasks were vented, swirled manually, and returned to the oven. Fermentation was terminated by placing the flasks in cold water (4°C).

Substrates

The two substrates used were:
- Coastcross hay (89.2% dry mater [DM], 9.7% crude protein [CP], 1.3% ether extract [EE], 7.9% ash, 60.2% neutral detergent fibre [NDF], and 30.6% acid detergent fibre [ADF]);
- 80:20 concentrate:forage diet (20.0% Coastcross hay, 62.7% ground corn, 15.0% soybean meal, 1.0% limestone, and 1.3% mineral premix on DM basis; 91.5% DM, 15.7% CP, 3.3% EE, 4.3% ash, 20.3% NDF, and 8.8% ADF). The diet was formulated to meet the NRC (2007) recommendations for growing lambs by using the Small Ruminant Nutrition System v.1.8.0 (Cannas et al., 2004).

Both substrates were ground by using a Wiley mill (Marconi, Piracicaba, SP, Brazil) to pass a 1 mm screen. The DM was determined by oven drying at 105°C for 24 h, and OM after ashing at 550°C for 4 h (AOAC, 1990). Ether extract was also determined according to AOAC (1990). The CP (N × 6.25) was determined using a Leco FP528 (Leco Corporation, St. Joseph, MI, USA) combustion nitrogen analyser (AOAC, 1997). Concentrations of dietary NDF and ADF were ash-corrected and determined by the non-sequential method using beakers according to Van Soest et al. (1991) and Goering and Van Soest (1970), respectively. The NDF analysis was performed with the addition of heat stable α-amylase (Ankom Technology, Tecnoglobo Equipamentos, Curitiba, Brazil) and sodium sulfite.

Inocula Preparation

The inocula from sheep on pasture were obtained using three rumen cannulated Santa Inês wethers (50 kg BWt) kept on pastures of signalgrass (*Brachiaria decumbens*) and elephantgrass (*Pennisetum purpureum*) with free access to a mineral premix and fresh water. Each animal was supplemented with 150 g/d of ground corn, 65 g/d of soybean meal, and 4.5 g/d of molasses. The inocula from feedlot sheep were obtained from a further three rumen cannulated Santa

Inês wethers (50 kg BWt) that were penned and individually fed 1.2 kg/d of the high concentrate diet described previously. Feed was provided twice daily in equal portions. Penned animals also had free access to a mineral premix and fresh water. Adaptation to the feeding conditions lasted at least 15 d. Ruminal liquid and solid fractions were collected independently before morning feeding and kept in pre-warmed thermos flasks under anaerobic conditions. Similar volumes (50:50 v/v) of both fractions were mixed in a blender for 10 s, squeezed with two layers of cheesecloth, and maintained in a water bath (39°C) under CO_2 flushing until the moment of incubation.

Monensin Sodium Solution

A stock solution of MON (M5273 – Sigma Aldrich Inc.) was prepared by diluting 15.6 mg MON (MWt = 692.85) in 1 mL of pure ethanol and preserved at −10°C until use. In each incubation flask (liquid volume 75 mL), 10 mL of the stock solution were added just before inoculation in order to achieve a final MON concentration of 3 mM (2.08 mg/L). According to Selje-Assman et al. (2008), 11.25 µL of ethanol in 75 mL of buffered rumen fluid had no measurable effects on fermentation. Ethanol was not included in the Control.

Laboratory Analyses and Calculations

Total gas production was estimated according to the equation:

$$V = 7.365 \times p \ (n = 500; r^2 = 0.99$$

data not published) where V is the gas volume (mL) and p is the measured pressure (psi).

After incubation, the pH of buffered rumen fluid was measured with a pH meter (Digimed DM21, São Paulo, SP, Brazil). The truly degraded DM (TDDM) was determined including in each flask 70 mL of neutral detergent solution (Van Soest et al., 1991) without α-amylase and incubating at 105°C for 3 h. The residue was filtered in pre-weighed crucibles, washed with hot water and acetone, being oven dried at 105°C for 16 h. The truly degraded OM (TDOM) was determined by ashing at 550°C for 4 h. The TDDM and TDOM were corrected for the DM and OM residues of the blanks. The partitioning factor (PF), expressed as the ratio of TDOM (mg) to the volume of gas produced (mL), was used to estimate the efficiency of microbial production (Blümmel et al., 1997).

Apparent DM and OM degradabilities were determined according to Getachew et al. (2000). The content of each flask was transferred into a pre-weighed (60°C for 24 h) centrifuge bottle (Du Pont Company, Wilmington, DE, USA) and centrifuged (Sorvall Superspeed RC2-B, Newton, CT, USA) at 23 000 g for 15 min. The supernatant was stored at −18°C until analysed for short-chain fatty acids (SCFA) and ammonia. The incubation flask was washed twice with 30 mL saline solution (0.9% NaCl), transferring the washing solution to the respective centrifuge bottle. Centrifugation was repeated and the bottle containing the pellet was frozen at −18°C and lyophilised (Labconco ThermoSavant Modulyo D–115, Holbrook, NY, USA). Apparent DM and OM degradabilities were determined by difference considering the weight of the centrifuge bottle containing the pellet minus the weight of the empty bottle and correcting for the pellet weight of the blanks.

The SCFA were determined by gas-liquid chromatography (GC HP 5890 Series II/ integrator HP 3396 Series II/automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA) according to Palmquist and Conrad (1971). The internal standard was 2-metylbutyric acid. Each tube contained 100 mL of internal standard, 800 mL of sample, and 200 µL of formic acid. A mixture of SCFA with known concentrations was used as external standard for the integra-

tor calibration. The ammonia concentration was determined using a microKjeldahl steam distillator according to Preston (1995). A 1 mL supernatant aliquot was distilled with 10-mL 5% sodium tetraborate solution and the ammonia released collected in 30-mL 20% boric acid solution and immediately titrated using 0.01 N sulphuric acid.

Methane concentration was determined by gas chromatography injecting 1 ml of gas in a Shimadzu 2014 GC (Shimadzu, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1/16″ OD, 1.0 mm ID, 1 m length; Ref. No. 19809, Restek, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionisation detector were 60 °C, 200 °C, and 240 °C, respectively. Helium at 10 mL/min was used as the carrier gas. In order to calculate the CH_4 concentration, a standard curve (0, 3, 6, 9, and 15% of CH_4) was prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 99.5% purity). Methane production (mL) was calculated by multiplying the total gas volume plus 85 mL (headspace) by the CH_4 concentration and correcting for the CH_4 produced by the blanks (Longo et al., 2006).

Plant Description and Essential Oil Characterisation

C. verbenacea (leaves and stems) was cultivated at the Chemical, Biological and Agricultural Research Center, State University of Campinas, Campinas, SP, Brazil. The EO was obtained by hydrodistillation in boiling water for 4 h by using a Clevenger device and a condenser column. The EO separation was performed by density difference using a separation funnel and filtrating with anhydrous sodium sulphate to remove residual water. The identification of the compounds present in the EO was done by GC-MS (GC HP 6890; mass selective detector HP 5975; automatic injector HP 7673, Agilent Technologies, Palo Alto, CA, USA) using a HP–5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm; stationary phase 5% methyl silicone). Helium was used as the carrier gas (1.0 mL/min flow rate). The mass spectrum was acquired by electron impact ionisation (scan mode) at an electron energy of 70 eV. Samples (1 μL) were injected in the split mode employing a split ratio of 1:40. The temperature column programme was 110 °C/2 min followed by heating to 300 °C (5 °C/min). Injector and detector temperature were 220 °C and 250 °C, respectively. The essential oil was diluted in ethyl acetate (15 mg/mL) before injection. The compounds were identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) system data bank with a minimum of 95% of concordance when compared with the literature (Adams, 2001). A standard solution of n-alkanes was co-injected with the sample in order to calculate the retention index and provide additional identification criteria.

Statistical Analysis

Data were analysed by the Proc MIXED (SAS Inst. Inc., Cary, NC) considering treatment as fixed effect and inoculum (n = 2) as random effect. The two substrates (hay and high concentrate diet) were analysed independently. Means were obtained by using the LSMEANS option and differences were declared significant by using the Tukey ANOVA test at P < 0.05.

RESULTS AND DISCUSSION

Essential Oil Composition

The major components of the EO from *C. verbenacea* were: 28.19% of *trans*-caryophyllene, 23.58% of alpha-pinene, 6.90% of aloaromadendrene, and 4.54% of alpha-humulene (**Table 1**). A similar composition of *C. verbenacea* EO has been previously published (Carvalho Jr. et al., 2004). Considering the major compounds in

the *C. verbenacea* EO, alpha-pinene was the only one that had been previously tested on ruminal fermentation. According to Busquet et al. (2006), the addition of cade oil (*Juniperus oxycedrus*) containing 35% of alpha-pinene resulted in small effects on *in vitro* ruminal fermentation, arguing that the lack of effect was probably related to the low oxygenated hydrocarbons content in this compound.

Effect on Coastcross Hay Fermentation

The effects of MON and *C. verbenacea* EO on 24-h *in vitro* fermentation of Coastcross hay are shown in **Table 2**. Gas production, expressed as mL/g of DM or $OM_{incubated}$, was reduced (P < 0.05) by MON when compared with the Control, mainly because substrate degradation was depressed. The reduction in TDDM and TDOM (P < 0.05) recorded for MON is not considered a negative effect and represents a basic limitation of short-term *in vitro* experiments (Russell and Strobel, 1988). Monensin inhibits cellulolytic ruminococci and also a cellulolytic strain of *Butyrivibrio fibrisolvens,* but *Fibrobacter succinogenes*, another cellulolytic species, is able to grow in the presence of MON (Chen and Wolin, 1979). However, *F. succinogenes* has a long growth lag time and only under *in vivo* conditions is this species able to replace the sensitive species of cellulolytic bacteria (Russell and Strobel, 1988). The MON also reduced (P < 0.05) gas production expressed/unit of $DM_{degraded}$, which is consistent with the greater propionate concentration (P < 0.05) and PF value (P < 0.05) when compared with the Control. According to the stoichiometry of gas production, propionate formation is always associated with lower gas production (Cone, 1998; Makkar, 2004).

The COR37.5 showed no effect on gas production when expressed as mL/g DM or $OM_{incubated}$. However, TDDM and TDOM were affected negatively (P < 0.05) by COR37.5, showing that *C. verbenacea* EO inhibited the activity of rumen microorganisms. As a result, gas production expressed as mL/g DM or $OM_{degraded}$, was increased (P < 0.05) and PF value was reduced (P < 0.05) by COR37.5. These results indicate that the microbial production efficiency was reduced and less degraded matter was incorporated into microbial mass (Blümmel et al., 1997). The COR75 showed even more pronounced effects than COR37.5. Despite the reduction in degradability caused by both MON and EO, the most important effect was that MON increased (P < 0.05) whereas EO reduced (P < 0.05) the PF value when compared with the Control. This indicates that utilisation of EO from *C. verbenacea* may not benefit ruminant animals.

Methane production, expressed as mL/g DM or $OM_{incubated}$, showed a 48% reduction (P < 0.05) when MON was compared with the Control. Reductions of 48, 52, and 58% respectively were reported using hay as substrate when 2.5, 5.0, and 12.5 mg/L of MON where added *in vitro* (Russell and Strobel, 1988). This demonstrates that very high doses of MON do not promote further reductions in CH_4 production. The dose of EO used in COR37.5 was too low to produce any detectable effect on CH_4 production. However, CH_4 produced/unit DM or $OM_{incubated}$ was reduced (P < 0.05) by 30% when COR75 was compared with the Control. Although not statistically different, intermediary values of CH_4 production expressed as mL/g $OM_{degraded}$ were observed for COR75 (32.9) when compared with the Control (38.9) and MON (25.8). Thus, it is speculated that higher doses of EO from *C. verbenacea* may reduce CH_4 production as much as MON. To support this idea, a previous study showed that peppermint (*Mentha piperita*) oil progressively inhibited *in vitro* methanogenesis by 19.9, 46.0, and 75.6% at levels of 0.33, 1.0, and 2.0 μL/mL, respectively (Agarwal et al., 2009).

Compared with the Control, total SCFA and acetate concentrations were not affected by MON, COR37.5 or COR75. However, the lower total SCFA (69.47 mM vs. 73.81 mM) and acetate (50.96

Table 1. Major volatile compounds identified by GC-MS analysis of the *Cordia verbenacea* **D.C. essential oil**

RT (min)[1]	RI[2]	Compound[3]	Relative %[4]
4.95	927	Alpha-Tujene	1.41
5.18	936	Alpha-Pinene	23.58
5.47	948	Camphene	0.19
6.10	973	Sabinene	0.99
6.19	977	Beta-Pinene	1.07
6.54	992	Beta-Myrcene	0.52
7.72	1 029	Beta-Felandrene	1.20
7.79	1 031	1,8-Cineole	1.76
17.64	1 284	Bornile acetate	0.77
19.79	1 336	Delta-Elemene	1.55
20.50	1 353	n.i.	0.31
21.34	1 373	Alpha-Copaene	0.80
21.95	1 388	Beta-Cubebene	0.46
22.04	1 390	Beta-Elemene	1.32
22.70	1 407	Alpha-Cedrene	2.23
23.18	1 418	*trans*-Caryophyllene	28.19
23.64	1 430	Beta-Gurjunene	0.75
23.81	1 434	Alpha-*trans*-Bergamotene	0.37
24.13	1 442	Beta-(Z)-Farnesene	0.42
24.51	1 452	Alpha-Humulene	4.54
24.69	1 456	Beta-(E)-Farnesene	2.55
24.82	1 459	Aloaromadendrene	6.90
25.59	1 478	Germacrene D	2.07
25.76	1 483	n.i.	1.11
26.21	1 494	Bicyclogermacrene	2.71
26.54	1 502	Alpha-(Z)-Bisabolene	1.66
26.75	1 508	Beta-Bisabolene	3.02
27.19	1 519	n.i.	0.64
27.29	1 522	Delta-Cadinene	2.03
27.62	1 530	Gamma-(E)-Bisabolene	1.08
29.48	1 579	Caryophyllene oxide	0.93
32.77	1 668	n.i.	1.42
33.06	1 676	n.i.	1.47

[1] — Retention time; [2] — Retention index; [3] n.i. — non-identified; [4] = percentage relative to the total area integrated in the chromatogram.

mM vs. 54.29 mM) concentrations recorded for MON compared with the Control is related to the reduction on apparent DM degradability. A decrease in acetate concentration was expected because gram-positive bacteria, which mainly produce acetate, are sensitive to MON (Russell and Houlihan, 2003). Propionate concentration was increased (P < 0.05) by MON even with hay as substrate. In contrast, this same variable was reduced (P < 0.05) by COR37.5 and COR75 when compared with the Control. The inhibition of methanogenesis observed for MON is always coupled with an increase in propionate and a decrease in acetate concentrations (Russell and Strobel, 1989), but this was not recorded when *C. verbenacea* EO was used. It might therefore be speculated that this EO may affect methanogens directly since the mode of action of MON is indirect, with CH$_4$ being reduced by inhibiting hydrogen and formate-producing bacteria (Russell and

Strobel, 1989). A previous *in vitro* trial showed that peppermint oil inhibited methanogenic microorganisms directly, and CH$_4$ production was reduced even with a decrease in propionate concentration and an increase in the acetate:propionate ratio (Agarwal et al., 2009).

The acetate:propionate ratio was decreased (P < 0.05) by MON mainly due to the reduction recorded in acetate concentration. In contrast, the ratio was not affected by COR37.5 or COR75. All other SCFAs were reduced (P < 0.05) by MON compared with the Control, with the exception of valerate. Butyrate is generally reduced by MON because this ionophore inhibits the major butyrate producer, the gram-positive bacteria *Butyrivibrio fibrisolvens* (Russell and Strobel, 1989). The reduced concentrations of iso-acids are indicative of lower deamination, since iso-acids are derived from catabolism of branched-chain amino acids (Mackie and White, 1990). In the case of

Table 2. Effect of monensin (3 μM) and *Cordia verbenacea* D.C. essential oil (37.5 or 75 μL in 75 mL of buffered rumen fluid) on 24-h *in vitro* fermentation of Coastcross hay.

Item[1]	Treatments[2]				SEM[3]
	Control	MON	COR37.5	COR75	
Gas					
mL/g $DM_{incubated}$	117.3^a	78.4^c	119.4^a	106.0^b	1.9
mL/g $OM_{incubated}$	125.6^a	83.9^c	127.9^a	113.5^b	2.0
mL/g $DM_{degraded}$	228.9^b	206.0^c	252.7^a	255.7^a	5.5
mL/g $OM_{degraded}$	252.7^b	230.7^b	278.5^a	284.9^a	5.9
TDDM (%)	49.58^a	39.21^c	45.85^b	41.91^c	0.79
TDOM (%)	48.08^a	37.51^c	44.54^b	40.26^c	0.71
ApDDM (%)	33.67^a	28.30^c	31.74^{ab}	29.48^{bc}	1.11
Partitioning factor	3.97^b	4.42^a	3.60^{bc}	3.57^c	0.10
Methane					
mL/g $DM_{incubated}$	17.5^a	9.0^c	15.9^a	12.2^b	0.7
mL/g $OM_{incubated}$	18.7^a	9.7^c	17.0^a	13.1^b	0.7
mL/g $DM_{degraded}$	35.2^a	23.0^b	34.7^a	29.5^{ab}	1.8
mL/g $OM_{degraded}$	38.9^a	25.8^b	38.2^a	32.9^{ab}	2.0
SCFA, mM					
Total	73.81^{ab}	69.47^b	74.42^a	73.32^{ab}	1.13
Acetate	54.29	50.96	55.13	54.36	1.11
Propionate	9.88^b	10.16^a	9.57^c	9.27^d	0.05
Isobutyrate	0.55^a	0.43^b	0.57^a	0.56^a	< 0.01
Butyrate	7.39^a	6.38^b	7.44^a	7.31^a	0.06
Isovalerate	1.11^b	0.98^c	1.19^a	1.19^a	0.01
Valerate	0.60	0.57	0.53	0.63	0.05
Acetate:propionate	5.50^a	5.02^b	5.76^a	5.86^a	0.12
NH_3, mg/100 mL	26.49^b	27.63^{ab}	30.46^a	27.91^{ab}	0.89
pH at 24 h	6.70^c	6.76^{ab}	6.73^b	6.77^a	< 0.01

[1] TDDM = truly degraded dry matter; TDOM = truly degraded organic matter; ApDDM = apparently degraded dry matter; Partitioning factor = mg $OM_{degraded}$/mL $gas_{produced}$; SCFA = short-chain fatty acids.

[2] Means followed by distinct letters within row differ by Tukey test ($P < 0.05$).

[3] SEM = standard error of the mean.

COR37.5 and COR75, isobutyrate, butyrate, isovalerate, and valerate concentrations did not differ from the Control.

Despite the reduced iso-acids concentrations, ammonia concentration was not reduced by MON as expected. *In vitro* studies have shown that MON decreases deamination (Russell and Strobel, 1989) and also inhibits a group called hyper-ammonia-producing bacteria which have a high specific activity for ammonia production (Russell et al., 1988). The lack of effect on ammonia concentration may be explained by the limited microbial growth and excessive microbial lyses which result in the high ammonia concentrations commonly observed for *in vitro* conditions (Cone, 1998). When the EO was used, ammonia concentration was not affected by COR75 but was increased (P < 0.05) by COR37.5. There is no clear explanation for this finding.

The pH value after 24-h incubation was increased (P < 0.05) by MON and EO inclusion. Higher pH values have been observed with MON addition and are generally attributed to the inhibition of lactate-producing bacteria (e.g. *Streptococcus bovis*; Russell and Strobel, 1989). Unfortunately, lactate concentration was not

measured. The lower pH observed for the Control is also consistent with the greater TDOM verified for this treatment compared with the other treatments. However, the effect on pH must be carefully interpreted, since *in vitro* pH is controlled by buffering agents.

Effect on Fermentation of Concentrate Diet

The effects of MON and *C. verbenacea* EO on 16-h *in vitro* fermentation of the 80:20 concentrate:forage diet are shown in **Table 3**. In general, the doses of EO tested seemed to be too low to produce any effect on fermentation of the high concentrate diet. The interaction of EO and type of substrate was not considered statistically, but some differences occurred on the EO effect using hay or high concentrate diet, especially on CH_4 production. Interestingly, a previous study did not detect significant interaction between a commercial blend of EO and the type of diet (high concentrate or high forage) on DM degradation, SCFA profiles, and N metabolism in continuous culture fermentation (Castillejos et al., 2005).

Table 3. Effect of monensin (3 μM) and *Cordia verbenacea* **D.C. essential oil (37.5 or 75 μL in 75 mL of buffered rumen fluid) on 16-h** *in vitro* **fermentation of an 80:20 concentrate:forage diet.**

Item[1]	Treatments[2]				SEM[3]
	Control	MON	COR37.5	COR75	
Gas					
mL/g $DM_{incubated}$	213.1[a]	199.8[b]	212.5[a]	206.5[ab]	3.0
mL/g $OM_{incubated}$	223.0[a]	209.0[b]	222.4[a]	216.0[ab]	3.2
mL/g $DM_{degraded}$	278.5[ab]	268.4[b]	288.3[a]	284.4[ab]	4.6
mL/g $OM_{degraded}$	290.5[ab]	279.3[b]	300.0[a]	298.4[a]	4.6
TDDM (%)	76.67[a]	73.68[ab]	73.28[ab]	71.03[b]	0.99
TDOM (%)	76.89[a]	74.08[ab]	73.68[ab]	70.83[b]	0.98
ApDDM (%)	44.74[c]	56.03[ab]	48.52[bc]	59.64[a]	2.15
Partitioning factor	3.48[ab]	3.59[a]	3.35[b]	3.36[b]	0.05
Methane					
mL/g $DM_{incubated}$	31.1[a]	22.8[b]	30.3[a]	31.9[a]	1.1
mL/g $OM_{incubated}$	32.5[a]	23.9[b]	31.7[a]	33.4[a]	1.2
mL/g $DM_{degraded}$	40.6[a]	31.1[b]	41.4[a]	45.0[a]	1.8
mL/g $OM_{degraded}$	42.3[a]	32.4[b]	43.1[a]	47.2[a]	1.9
SCFA, mM					
Total	90.43[ab]	91.22[a]	83.83[b]	93.32[a]	1.63
Acetate	56.78[a]	55.78[ab]	50.33[b]	58.26[a]	1.43
Propionate	17.08[b]	21.05[a]	16.55[b]	17.36[b]	0.34
Isobutyrate	1.27[a]	1.11[b]	1.28[a]	1.33[a]	0.02
Butyrate	11.17[b]	9.52[c]	11.54[ab]	12.04[a]	0.16
Isovalerate	3.08[ab]	2.76[b]	3.10[ab]	3.23[a]	0.09
Valerate	1.06[ab]	0.98[b]	1.03[ab]	1.10[a]	0.02
Acetate:propionate	3.34[a]	2.66[b]	3.06[a]	3.37[a]	0.09
NH_3, mg/100 mL	45.19	54.26	53.52	52.77	2.64
pH at 16 h	6.55[a]	6.53[b]	6.57[a]	6.56[a]	< 0.01

[1] TDDM — truly degraded dry matter; TDOM — truly degraded organic matter; ApDDM — apparently degraded dry matter; Partitioning factor — mg $OM_{degraded}$/mL $gas_{produced}$; SCFA — short-chain fatty acids.

[2] Means followed by distinct letters within row differ by Tukey test (P < 0.05).

[3] SEM — standard error of the mean.

As verified for hay, gas production expressed per unit of DM or $OM_{incubated}$ was reduced (P < 0.05) by MON. However, this effect occurred to a lesser extent than verified for hay, which is explained by the similar values of TDDM and TDOM between Control and MON. Although not statistically different, the lower values of gas production expressed as mL/g DM or $OM_{degraded}$ are consistent with the greater propionate concentration recorded for MON compared with the Control. The inclusion of *C. verbenacea* EO at the two doses tested did not affect gas production variables.

The TDDM and TDOM were not affected by MON when compared with the Control, a result consistent with the observation that iono-phores do not decrease starch digestion. The COR37.5 also did not affect TDDM and TDOM, but a reduction (P < 0.05) was observed for COR75 compared with the Control. It is important to highlight that the overestimation of TDDM and TDOM (i.e. starch solubilisation) by the utilisation of neutral detergent solution seemed to be negligible. Using the same conditions of this experiment, a previous trial deter-mined that the undegraded residue after 16-h incubation contained only 5.96% starch (data not published). Unfortunately, the amount of starch solubilised by the addition of neutral detergent solution was not determined; nevertheless it is possible to assume that any inter-ference on degradability estimation was very low, because calculated PF values of all treatments were between the theoretical PF range of 2.74 to 4.65 (Blümmel et al., 1997). Compared with the Control, PF values were not affected by MON or EO.

Methane production, expressed as mL/g $DM_{incubated}$ and mL/g $DM_{degraded}$, was reduced (P < 0.05) by 27% and 23% respectively when MON was compared with the Control. Using a similar MON concentration and corn meal as the substrate, a previous study reported a 32% decrease in CH_4 production with MON addition (Russell and Strobel, 1988). In contrast to the data obtained for hay, CH_4 production (all variables) was not affected by inclusion of *C. verbenacea* EO when the high concentrate diet was used as substrate. While it is known that some EO effects on rumen fermentation are pH-dependent (Cardozo et al., 2005), this idea is not plausible under the present experimental conditions because the *in vitro* pH was buffer-controlled.

Total SCFA and acetate concentrations did not differ between Control and MON. However, MON resulted in greater (P < 0.05) propionate concentrations, leading to lower (P < 0.05) acetate:propionate ratios when compared with the Control. The gram-negative bacteria in the rumen, which mainly produce propionate and succinate, are MON-resistant (Russell and Houlihan, 2003), which explains the increase in propionate concentration. It is important to note that the acetate:propionate ratio decreased in the hay fermentation because acetate concentration was reduced, whereas this ratio decreased in the high concentrate diet fermentation due to the greater concentration of propionate. As verified for hay, MON reduced (P < 0.05) the concentrations of butyrate and iso-acids, without affecting valerate. Once again, ammonia concentrations did not differ between MON and the Control, and no effect was recorded on SCFA variables when COR37.5 or COR75 were compared with the Control. The only exception was for butyrate, which had a greater concentration for COR75 than the Control. Surprisingly, COR37.5 resulted in lower total SCFA and acetate concentrations than COR75. As verified for MON, ammonia concentration was not affected by COR37.5 or COR75. This result indicates that EO inclusion was not effective in reducing deamination, although some effect may occur in the processes of proteolysis or peptidolysis. Moreover, a period of adaptation to the rumen environment is generally necessary to observe PSM effects on N metabolism (Calsamiglia et al., 2007), and such effects would not be possible to evaluate under short-term *in vitro* incubations.

CONCLUSIONS

The EO from *Cordia verbenacea* D.C. was able to modify *in vitro* ruminal fermentation. The most promising effect was related to the inhibition of methanogenesis using hay as substrate. This experiment indicates that doses greater than 1 µL/mL of buffered rumen fluid may decrease CH_4 production as much as MON. However, the negative effect on the reduction of microbial production efficiency should be carefully considered. No positive effects were observed with the inclusion of *C. verbenacea* EO when the 80:20 concentrate:forage diet was used as substrate. However, taking into account the results obtained for hay, doses greater than 1 µL/mL of buffered rumen fluid should also be evaluated under the high concentrate diet condition.

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Reduction in Methane Emissions from Ruminants by Plant Secondary Metabolites: Effects of Polyphenols and Saponins

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ABSTRACT

The effects of plant secondary metabolites (PSM), specifically polyphenols (tannins) and saponins on rumen fermentation and methanogenesis were investigated using the Hohenheim gas method. We evaluated the effects of: (1) polyphenol-containing plants, (2) simple phenols in the form of phenolic acids, (3) purified tannins, (4) saponin-containing plants, and (5) isolated saponin-rich fractions on rumen methanogenesis. Statistically significant negative relationships between total phenols, total tannins or tannin activity and methane (CH_4) production were observed, whereas no correlation existed between condensed tannins and CH_4 production. Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH_4 production significantly when added at 5 mM. Addition of purified chestnut and sumach tannins (hydrolysable tannins) at 1 mg/mL to the *in vitro* rumen fermentation system containing hay:concentrate (70:30) decreased CH_4 production ($P < 0.05$), by 6.5 and 7.2% respectively. However, addition of mimosa and quebracho tannins (condensed tannins) at this concentration did not decrease CH_4 production. For studying the effects of saponins, leaves of *Sesbania, Knautia* and seeds of Fenugreek, and their saponin-rich fractions were evaluated. Addition of Fenugreek and *Sesbania* plant materials to hay or the hay-concentrate mixture increased partitioning factor (PF, expressed as mg truly degraded substrate/mL gas produced; a measure of efficiency of microbial protein synthesis) and decreased CH_4 production per unit substrate degraded. These plant materials and their saponin-rich fractions did not reduce CH_4 production in absolute amounts despite decreases in protozoal numbers by 40–50%. The saponins altered the microbial community towards proliferation of fibre-degrading bacteria and inhibition of fungal population. The results with saponin-containing plant materials and their isolated fractions indicated a weak association between anti-protozoal activity of saponins and methanogenesis. Nevertheless, the saponin-containing plants possess potential to partition higher proportions of the substrate to microbial mass production

INTRODUCTION

The emission of greenhouse gases such as carbon dioxide and CH_4 is considered to be one of the most important global environmental issues (IPCC, 2001). Animals, particularly ruminants, produce CH_4 from anaerobic fermentation in their gastro-intestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism. Ruminant livestock are responsible for about 15–20% of the total anthropogenic emission of CH_4 (Moss et al., 2000). The CH_4 produced from enteric fermentation of ruminants is not only related to environmental problems, but is also associated with energy losses and, hence reductions in their retention and use of energy. Typically 6–8%, but up to 12%, of the gross energy (GE) in feed is converted to CH_4 during microbial digestion in the rumen (Johnson and Johnson, 1995). Therefore, decreasing CH_4 production from ruminants is desirable for reducing greenhouse gas emissions and increasing utilisation of the digested energy. Plant secondary metabolites (PSM) have been suggested as effective alternatives to antibiotics to suppress rumen methanogenesis through their antimicrobial activity (Makkar et al., 2007; Jayanegara et al., 2009). Plant secondary metabolites constitute the group of chemicals present in plants that are not involved in the primary biochemical processes of plant growth and reproduction. The potential of these compounds and specifically of polyphenols (tannins) and saponins to reduce enteric CH_4 production has been recognised and extensive screening of a large range of plants and their secondary compounds is underway in several laboratories. The antimicrobial action and effects on rumen fermentation of these compounds depend on their nature, activity and concentration. We present here work conducted in our laboratory on the potential of various polyphenols (tannins), saponin-rich plants and isolated saponin-rich fractions to reduce CH_4 emission from ruminants.

RESULTS AND DISCUSSION

Polyphenols

The evaluation of polyphenols was conducted using the *in vitro* Hohenheim gas production method (Menke and Steingass, 1988) as modified by Makkar et al. (1995). We examined a number of polyphenol-containing plants, non-tannin simple phenolics, and purified tannins.

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Table 1. Correlation coefficients between tannin assays and *in vitro* rumen methane production (n = 17).

Assays[b]	Methane (ml/100 mL)	Decrease in CH_4 (%)	Increase in CH_4[a] (%)
TP	−0.59*	0.57*	0.78***
TT	−0.60*	0.54*	0.62**
CT	−0.07[ns]	0.09[ns]	0.24[ns]
Tannin bioassay	−0.75***	0.79***	0.92***

TP = total phenols; TT = total tannins; CT = condensed tannins.
[a] Methane increase on polyethylene glycol (MW 6 000) addition; [b] for assay protocols see Makkar (2003a).
[ns] not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2. Effect of addition of simple phenols on gas production, methane production and organic matter digestibility.

Treatment	Gas (mL)	CH_4 (mL/100 mL)	Decrease in CH_4 (%)	OMD (%)	CH_4/OMD (mL/100 mg)	Decrease in CH_4/OMD (%)
Control	76.2[c]	15.9[cd]	0.0[b]	76.1	5.05[bc]	0.0[ab]
Benzoic						
2 mM	77.0[c]	16.0[cd]	−0.6[ab]	75.2	5.19[c]	−2.8[a]
5 mM	74.8[bc]	16.0[cd]	−0.3[ab]	75.2	5.03[abc]	0.4[ab]
Cinnamic						
2 mM	75.3[bc]	15.5[abc]	2.6[bcd]	75.7	4.88[abc]	3.2[abcd]
5 mM	74.5[bc]	15.4[abc]	3.4[cde]	75.5	4.79[abc]	5.0[abcd]
Phenylacetic						
2 mM	73.3[abc]	15.9[bcd]	0.2[abc]	73.7	5.00[abc]	0.9[abc]
5 mM	74.3[bc]	16.4[d]	−3.1[a]	75.1	5.13[c]	−1.7[ab]
Caffeic						
2 mM	73.3[abc]	15.6[abc]	2.0[bcd]	73.2	4.94[abc]	2.1[abcd]
5 mM	71.0[ab]	14.9[a]	6.3[e]	73.4	4.57[a]	9.4[d]
p-Coumaric						
2 mM	72.5[abc]	15.5[abc]	2.4[bcd]	71.8	4.96[abc]	1.6[abcd]
5 mM	68.5[a]	15.1[a]	5.1[de]	71.0	4.61[ab]	8.5[cd]
Ferulic						
2 mM	72.5[abc]	15.9[bcd]	0.4[bc]	75.2	4.84[abc]	4.0[abcd]
5 mM	70.8[ab]	15.2[ab]	4.7[de]	71.4	4.77[abc]	5.5[bcd]
SEM	0.49	0.08	0.51	0.43	0.039	0.75

OMD = organic matter digestibility.
Values in the same column with different superscripts are different at $P < 0.05$.

Polyphenol-containing Plants

Using 17 polyphenol-containing plants (**Table 1**), statistically significant negative relationships between total phenols (TP), total tannins (TT) or tannin activity and CH_4 production existed, whereas the relationship between condensed tannins (CT) and CH_4 production was not significant. The highest correlation was found between tannin activity determined by the tannin bioassay and CH_4 decrease.

Since the correlations between TP and decrease in CH_4 or increase in CH_4 on addition of polyethylene glycol (a tannin-inactivating agent) were higher than those for TT, it seems that non-tannin phenols contribute to reducing CH_4 production. It would be interesting to obtain direct evidence by isolating non-tannin phenols and incubating them in the *in vitro* gas method. These results, if confirmed, could have wide application since non-tannin phenols are not likely to decrease the utilisation of proteins and other nutrients, but could also have beneficial effects (antioxidant, anticarcinogenic) associated with phenolic compounds (Makkar, 2003b; Makkar et al., 2007).

Although it was evident from these results that tannin-containing plants are able to reduce ruminal CH_4 emission, some reports suggest that tannins have no significant effect on rumen CH_4 production. For example, Oliveira et al. (2007) reported that there was no effect of tannin levels on CH_4 emission from diets containing sorghum silages. Beauchemin et al. (2007) also reported that feeding a diet containing an extract of quebracho tannins at a level up to 20 g/kg dry matter did not reduce enteric CH_4 emissions from growing cattle, although the protein-binding effect of the quebracho tannin extract was evident. The different results obtained using different tannins could be

attributed to their nature, structure or activity and to the concentrations at which they were used.

Non-tannin Phenolics

The above study indicated that non-tannin phenols play a role in CH_4 reduction. In the next study we evaluated six simple phenols (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids), as representatives of non-tannin phenols. All of these simple phenols were added at two different concentrations, i.e. 2 and 5 mM. The results are presented in **Table 2**.

In general, the addition of simple phenols decreased gas production although most of them were not significantly different and the effects were higher at higher concentrations. None of the simple phenols was effective in decreasing CH_4 production at the lower concentration (2 mM). Cinnamic, caffeic, p-coumaric and ferulic acids decreased CH4 production significantly (P < 0.05) when added at 5 mM. Caffeic acid at 5 mM was the most effective of the simple phenols tested, decreasing CH_4 by 6.3% compared with the control. The magnitude was higher (9.4% compared with the control) when expressed as decrease of CH_4 per unit organic matter digested. After caffeic acid, the order of simple phenols to decrease CH4 was: p-coumaric > ferulic > cinnamic. Phenolic acid containing tri-hydroxy group (caffeic acid) had a higher CH_4 inhibitory effect than those containing di-hydroxy groups (p-coumaric acid and ferulic acid). The phenolics containing a single hydroxy group (benzoic, phenylacetic and cinnamic acids) had the least effect. These results suggest that phenolics with higher numbers of hydroxyl groups are expected to elicit higher CH_4 inhibitory effects.

The effect of phenolic acids on methanogenesis could be expected since they affect the activities of rumen microbes. The decrease in ruminal CH_4 production could be linked to their role in inhibiting fibre degradation and in decreasing protozoa to a certain extent. Inhibition of fibre degradation will shift short chain fatty acid (SCFA) composition away from acetate and hence less production of hydrogen and less CH_4 formation. On the other hand, the anti-protozoal effect of phenolic acids would decrease CH_4 production since a portion of methanogens is attached to protozoa (Vogels et al., 1980). These protozoa-associated methanogens have been reported to contribute up to 37% of total rumen CH_4 emissions (Klieve and Hegarty, 1999). Therefore reduced protozoal counts in the rumen are associated with the reductions in CH_4 production, however, this is not always the case since a weak association between protozoal numbers and methanogenesis was observed with saponin-containing plants (discussed in later sections).

Phenolic acids are common constituents of forages fed to ruminants, where they occur most commonly as hydroxycinnamic acids ester-linked to polysaccharide. Ferulic and p-coumaric acids, the major phenolic acids found in this form, may represent up to 2.5% by weight of the cell walls of temperate grasses (Hartley and Jones, 1977). In the present study, phenolic acid concentrations of 2 and 5 mM were equal to 1.9–3.1 and 4.8–7.7% of the substrate dry matter incubated, respectively, depending on the structure and molecular weight of each phenolic acid. The lower concentration used was therefore in a reasonable range, while the higher concentration might also be in a reasonable range for the tropical forages, which generally contain higher concentrations of lignified tissues and secondary metabolites than temperate forages. In the in vivo situation, rumen microbes might encounter such a high concentration of phenolic acids provided that all phenolic acids are released from plant tissues, which is normally not the case. However, the microbes attached to the plant tissues are likely to encounter higher concentrations of phenolic acids in their microenvironment.
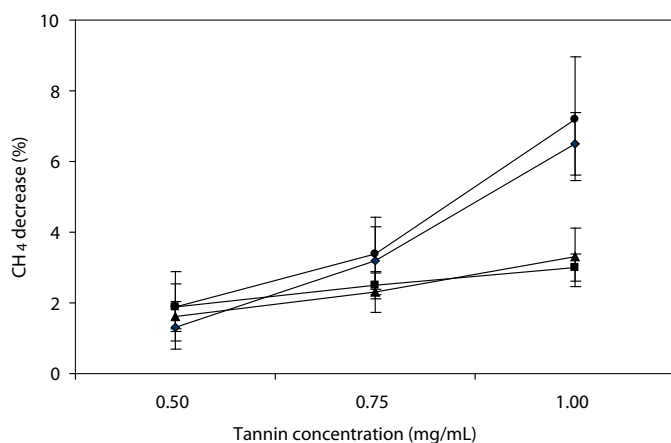
Purified Tannins

Some studies have reported that feeding tannin-containing forages to ruminants reduces CH_4 emissions (e.g. Puchala et al., 2005). However, in most of those studies, the reduction in CH_4 was confounded by changes in forage composition and quality. Lower fibre diets are associated with lower CH_4 emissions. Other nutrients such as lipid (oil) affect CH_4 production. Similarly, higher digestible feed is known to produce less CH_4 per unit feed intake. Negative effects on ruminal fibre digestion, which may relate to decreased number of cellulolytic bacteria, formation of tannin-cellulose complexes that are resistant to enzymatic digestion, and/or impaired substrate adhesion by fibrolytic microbes, would reduce hydrogen availability to lessen methanogenesis (Carulla et al., 2005). Thus, there is considerable uncertainty about the effectiveness of tannin-containing forages to reduce enteric CH_4 emissions from cattle. In the present study, therefore, other confounding components were omitted by using the purified tannins and, hence, specific effects of tannins were obtained. Different levels of purified tannins from chestnut, mimosa, quebracho and sumach (0.5, 0.75 and 1.0 mg/mL) were evaluated for their potential to reduce rumen CH_4 production. Chestnut and sumach tannins represented the hydrolysable tannins, whereas mimosa and quebracho tannins represented the condensed tannins.

The addition of purified chestnut and sumach tannins at 1 mg/ml to a hay:concentrate (70:30) diet significantly decreased (P < 0.05) CH_4 production by 6.5 and 7.2% respectively. Lower concentrations (0.5 and 0.75 mg/mL) did not significantly decrease CH_4 production. The addition of mimosa and quebracho tannins (condensed tannins) did not significantly decrease CH_4 production, even at the highest concentration. For all tannins, increases in concentration led to increases in CH_4 reduction (**Figure 1**). The condensed tannins decreased gas production and organic matter digestibility (OMD) more than the hydrolysable tannins. The results suggested that the hydrolysable tannins are more effective in decreasing CH_4 emissions than the condensed tannins, while at the same time the hydrolysable tannins did not significantly decrease OMD. The condensed tannins appear to decrease CH_4 more through reduced fibre digestion (indirect effect), while hydrolysable tannins act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The tannin concentrations of 0.5, 0.75 and 1.0 mg/mL were equal to 4.0, 5.9 and 7.9% of the substrate dry matter, respectively. Animals are likely to encounter such concentrations, especially in tropical regions, where they are exposed to high amount of tannins during the dry season. During the dry season, animals depend largely on fodder tree leaves and browses, and the tannin content in these feed resources is generally high (5–15%). However, it may be noted that the effects on the extent of CH4 reduction of tannins in the soluble form as in this study and in in vivo situations where tannins are a part of the feed might be different. Nevertheless, the in vitro studies give insight into the mechanism of action of various tannins, their comparative effects and possible in vivo effects.

Although it was evident from our research that polyphenols reduce rumen CH_4 production significantly, it should be noted that we used in vitro experiments to measure the effects. Flachowsky and Lebzien (2009) noted that it is extremely difficult to extrapolate from in vitro measurements to in vivo situations in ruminants, or to field conditions. This is because the relationship between CH_4 produced in vivo and in vitro is very poor (r^2 = 0.264). However, the in vitro studies should be considered as a starting point for screening of potential CH_4 inhibitors and should be combined with in vivo experiments. Therefore, Flachowsky and Lebzien (2009) proposed a three-step programme to assess the CH_4-reduction potential of feed additives or feeding measurements. This includes in vitro screening of substances,

Figure 1. Effect of purified tannins from chestnut (-♦-), mimosa (-■-), quebracho (-▲-) and sumach (-●-) on CH_4 decrease when added to hay:concentrate diet (70:30 w/w) at concentrations of 0.5, 0.75 and 1.0 mg/mL

short-term *in vivo* experiments in target animals and finally *in vivo* long-term recording of CH_4 production together with other animal performance parameters. Following this programme will substantially increase the relevance of such studies to the industry and potential users. Also, short term *in vivo* studies could also be replaced by continuous fermentation studies as conducted by Goel et al. (2009). This would save time and resources.

Saponins

Saponins are natural detergents, chemically defined as high molecular weight glycosides in which sugars are linked to a triterpene or steroidal aglycone moiety. These compounds possess membranolytic

properties, resulting in cell death by forming complexes with sterols on protozoal cell membranes (Cheeke, 1999). They modify ruminal fermentation by suppressing rumen protozoa and selectively inhibiting some bacteria. The symbiosis of protozoa with methanogenic bacteria in the rumen is well established and selective suppression of protozoa has been suggested to be a promising approach to reduce the CH_4 production (Dohme et al., 1999). Therefore, the plants rich in saponins have potential for enhancing flow of microbial protein from the rumen, increasing the efficiency of feed utilisation, and decreasing methanogenesis. We studied the effects of saponin-containing plants and their saponin-rich fractions for their anti-protozoal and CH_4 inhibition activities using the Hohenheim Gas Test (HGT). The first study was designed to observe the effect of saponin-rich plant materials on partitioning of nutrients from roughage- and concentrate-based feeds to CH_4, followed by another similar study with their isolated saponin-rich fractions. Fermentation parameters and microbial community structure were also investigated.

Saponin-containing Plants

The incubation of saponin-containing plant materials: leaves from *Sesbania* (*Sesbania sesban*) or seeds of Fenugreek (*Trigonella foenum-graecum* L.) as a sole substrate resulted in higher responses towards increasing the partitioning factors, PF (increasing efficiency of microbial mass synthesis) and increasing the reductions in CH_4 production (**Table 3a**). The plant materials when added to hay- or concentrate-based diets, did not produce substantial reductions in CH_4 production (**Table 3b**). The higher PF and CH_4 reductions were observed when the saponin-containing plant materials were added to concentrate-based diets. The crude plant extracts (in water and methanol/water (0.95:0.05, v/v) of the test plants had negligible effects on CH_4 production (data not shown). All different incubation materials: the sole plant material, plant material supplemented with hay- or concentrate-based diets or the plant extracts resulted in reductions in protozoal populations. However, these reductions did

Table 3. Effect of different plant materials on methane production.

a) Incubation with sole plant materials as substrate (380 mg/40 mL incubation fluid).

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate-hay fed animal				
Substrate	PF	MR (%)	MR$_{TD}$ (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR$_{TD}$ (%)	Protozoa (% reduction)
Hay	3.11a				Conc	3.16a			
Fenugreek	3.35a	-2.2	6.7a	68.1	Fenugreek	4.57b	20.5 b	29.1 a	73.2 b
Sesbania	4.63b	-3.4	30.4b	61.1	*Sesbania*	3.52a	5.4a	37.8 b	66.0 a
SEM	0.051	1.22	0.37	1.26	SEM	0.14	1.93	0.11	2.12

b) Supplementation of hay or concentrate (380 mg each) with the supplement (66 mg)

	Rumen liquor from roughage fed animal				Rumen liquor from concentrate - hay fed animal				
Substrate	PF	MR (%)	MR$_{TD}$ (%)	Protozoa (% reduction)	Substrate	PF	MR (%)	MR$_{TD}$ (%)	Protozoa (% reduction)
Hay	3.11[a]				Conc	3.16[a]			
Hay+F	3.28[a]	0.56	5.18[a]	49.5	Conc+F	3.33[ab]	4.86[b]	9.74[a]	56.0
Hay+S	3.45[a]	1.13	11.4[b]	47.8	Conc+S	3.52[b]	1.62 [a]	11.9[ab]	50.7
SEM	0.096	0.124	2.028	0.98	SEM	0.132	0.221	1.948	1.03

PF = partition factor (mg of substrate truly degraded/mL gas); MR = methane reduction on volume basis; MR$_{TD}$ = methane reduction on the basis of substrate truly degraded; F = Fenugreek seeds and S = *Sesbania* leaves.

Values in the same column with different superscripts are different at P < 0.05.

not accompany the decreases in CH_4 production in the incubations using rumen liquor from hay-fed animals; on the other hand, small reductions in CH_4 were produced in incubations with rumen liquor from concentrate-fed animals (P < 0.05). This observation indicates a weak association between the two parameters. The results from this study imply that the supplementations tested did not adversely affect the degradability of the basal feeds, hay or concentrate-hay mixture. Nevertheless, these plant materials when used as supplements, especially to the concentrate-based diet, have the potential to partition higher proportions of the substrate to microbial mass production and to elicit some CH_4 reduction per unit of substrate degraded. A weak association between protozoal number and methanogenesis was evident in this study and this association seemed to be diet dependent.

Saponin-rich fractions
Based on the results from saponin-containing plants, a further study was conducted with saponin-rich fractions prepared from the test plant materials: leaves of Sesbania (*Sesbania sesban*), Knautia (*Knautia arvensis*) and seeds of Fenugreek (*Trigonella foenum-graecum* L.). These fractions were evaluated for their effects on partitioning of nutrients from the concentrate-based diets to CH_4, SCFA and protozoa number. Additionally, changes in ammonia nitrogen (an important parameter in determining the N flow during substrate degradation which is used for microbial biomass production and absorption through the rumen cell wall), ammonia uptake

and microbial community structure were also studied using real-time PCR assay (Denman and McSweeney, 2006). The lower concentration of saponin-rich fractions used in this study corresponded to the quantity of raw material of the test supplements which showed promising response in the former study, except for *Knautia* leaves, which were not evaluated in the earlier study. Saponins have been reported to alter rumen fermentation by affecting the digestibility (either increase or no effect) and increasing microbial protein synthesis (Makkar et al., 1998). In the present study the fractions did not affect digestibility, and a trend towards slightly higher gas production was observed, which might be due to the saponin-mediated increase in fiber degrading bacteria (discussed below). Increase in the PF was not observed on supplementation of any of the saponin-rich fractions (**Table 4**), while increased PF values were observed on supplementation of the plant materials from which these saponins were isolated. Different responses have been observed for different saponins, which could be attributed to the different nature and/or concentration of saponins. Therefore, caution should be exercised in generalising the effects of saponins.

The maximum CH_4 reduction was observed for higher concentrations of saponin-rich fractions of *Sesbania* (6.1%) and *Knautia* (6.4%). Saponin-rich fractions were not as effective as the original plant material which when used as equivalent to the lower concentrations of saponin-rich fractions from Fenugreek seeds and *Sesbania* leaves decreased the protozoal count by nearly 50%, while this inhibition was 39% and 36% only by the corresponding saponin-

Table 4. Effect of saponin-rich fractions of test plants on methane production and protozoal numbers.

Substrate*	Partition Factor	MR%	MR$_{TD}$ (%)	Protozoa** ($\times 10^4$ mL/1)
S	3.25			19.54
S+F 5.62	3.12	1.82	−1.59	16.60 (15)
S+F 11.54	3.07	1.97	−4.47	11.93 (39)
S+Se 10.9	3.14	4.69	1.54	16.80 (14)
S+Se 21.8	3.08	6.14	1.71	12.41 (36)
S+K 3.88	3.16	5.50	3.23	16.83 (14)
S+K 7.76	3.16	6.43	3.94	14.66 (25)
SEM	0.122	1.821	1.112	1.224

*S = hay:concentrate (1:1), saponin-rich fractions (in mg) from: F = fenugreek; Se = *Sesbania*; K = *Knautia*. **Values in parentheses are the percentage reduction in protozoal number.

M — methane; MR — methane reduction on volume basis; TD — truly degraded substrate; MR$_{TD}$ — methane reduction on truly degraded substrate basis.

Table 5. Effects of saponin-rich fractions on SCFA and ammonia uptake.

Substrate*	Total SCFA (μmol m/L)	C2:C3	Branched SCFA (μmol m/L)	NH$_3$–Nitrogen (mg m/L)	NH3-uptake (mg NH$_3$ m/L gas)
S	871.6	3.63	15.61	0.37	0.0747
S+F 5.62	1014.0	3.76	17.79	0.37	ND
S+F 11.54	837.2	3.39	17.12	0.35	0.0934
S+Se 10.9	849.1	3.76	12.12	0.36	ND
S+Se 21.8	911.9	3.56	14.30	0.36	0.0618
S+K 3.88	866.3	3.60	13.76	0.37	ND
S+K 7.76	1035.2	3.77	11.82	0.37	ND
SEM	10.11	0.045	1.234	0.056	

* — as in Table 1; SCFA C2:C3 — acetate:propionate; ** — iso-butyrate + iso-valerate; ND — not determined.

rich fractions, as observed in the present study. This reduction in the activity of saponin-rich fractions could be due to non-extraction of all the saponins or to a change of saponin activity during their extraction.

No difference was observed in the SCFA production amongst control and saponin-containing incubations (**Table 5**). A slight decrease in ammonia concentration was observed only with Fenugreek (11.54 mg/ 40 mL) and *Sesbania* (10.9 and 21.8 mg/40 mL buffer) saponin-rich fractions. Wide variations of the effects of saponins on ammonia concentration have been reported in the literature. In a review by Wina et al. (2005), 14 reports indicate no effect of saponins on ammonia while another 17 studies state a negative correlation between saponin and ammonia production. The slight decrease in ammonia concentration might be due to high anti-protozoal activities of Fenugreek and *Sesbania* saponins at their higher concentrations. The lower number of protozoa results in lesser bacterial lysis and therefore lower release of breakdown products of protein. The rate of NH_3-N uptake (an index of the efficiency of microbial protein synthesis) was calculated as the slope of a linear regression between the amount of NH_3-N (in mg) and net gas (mL) (Getachew et al., 1998). The higher slope values on supplementation of saponin-rich fraction from Fenugreek (**Table 5**) suggest increased ammonia uptake by rumen microbes which means that the nitrogen from feed is converted into microbial protein to a greater extent in the presence of these saponins. But this increase in microbial protein was not reflected in the PF values, probably due to the measurement of PF at an inappropriate time (24 h in this study) and erosion of PF differences by microbial lysis (Blümmel et al., 2003).

Saponin-rich fractions changed the microbial population as estimated by the comparative delta Ct method (Denman and McSweeney, 2006). *Sesbania* saponins decreased methanogen population by 78%. Reduced rumen fungal populations (20–60%) and increases in *Fibrobacter succinogenes* (21–45%) and *Ruminococcus flavefaciens* (23–40%) were observed (**Figure 2**). In absolute terms, increases were observed in total bacterial population as indicated by decreased threshold cycle (Ct) values on supplementation of saponin-rich fractions. This effect was expected due to decrease in protozoal numbers since there is no predation of bacteria by protozoa (Matheiu et al., 1996). The increased populations of *F. scuccinogenes* can be attributed to their resistance to saponins as observed by Wang et al. (2000), suggesting that this species has the ability to deglycosylate and therefore inactivate saponins. Vinogradov et al. (2001) also
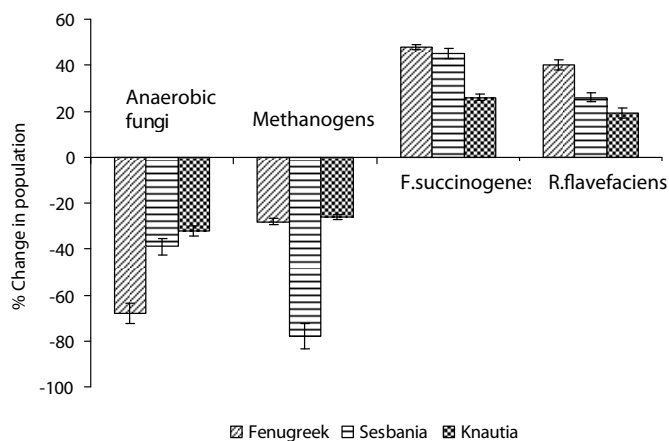
reported that the presence of 2-aminoethylphosphoric acid and glycolipid in the membrane enhances the membrane stability of *F. succinogenes*. Saponin-rich fractions were inhibitory to ruminal fungi as well. The inhibition of protozoal population also resulted in inhibition of methanogens though the effect was more pronounced for saponin-rich fraction of *Sesbania*. The Fenugreek fraction being more inhibitory to protozoa did not result in higher suppression of methanogens, which again reconfirms the weak association between the protozoal population and methanogen numbers.

Results for the effects of saponin-rich fractions on methanogens and CH4 levels were unexpected. These fractions decreased protozoa numbers and methanogen populations but did not decrease CH_4 production. The association between methanogens and protozoa is not obligatory and there is considerable evidence that different groups of methanogens are not equally associated with ciliate protozoa. A weak relationship between methanogenesis and the methanogen population expressed as a proportion of total anaerobes was observed by Nollett et al. (1998) under both *in vitro* and *in vivo* conditions. In our study, the lack of inhibition of CH_4 production with decreases in methanogens could have been caused by (i) a slow rate of association between protozoa and methanogens due to higher generation time of protozoa as compared with methanogens, (ii) an increased metabolism of methanogenic microbes independent of species remained after addition of saponins, and/or (iii) by changes in composition of the methanogenic community (Machmüller et al., 2003) and their increased efficiency of CH_4 production. The two major groups of methanogens in rumen: methanobacteriaceae (99.1% of total archea associated with protozoa) and free living methanobacteriales (0.05%) differ in their physiological characteristics. Therefore, based on the present results, it is suggested that on inhibition of protozoa, the species belonging to methanobacteriaceae declined with an increase in the number of free-living methanobacteriales. The reduced rate of association of protozoa and methanogens could result in higher interspecies hydrogen transfer between increased population of both hydrogen producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living methanobacteriales indicating no effect on CH_4 production.

CONCLUSIONS

Polyphenol or tannin containing plants decreased CH_4 production and, therefore, could be strategically used in diets for decreasing CH_4 emissions from ruminants. Amongst the tannin assays, tannin bioassay (a reflection of tannin activity) was the best predictor of the CH_4 reduction potential of a plant. Total phenols and total tannins were also good predictors of the CH_4 reduction potential. Methane decrease by addition of phenolic acids was relatively small (up to 6.3%), and the effect of phenolic acids on CH_4 reduction depended on the source and concentration applied. The order of simple phenols to decrease CH_4 was: caffeic acid > p-coumaric > ferulic > cinnamic. Hydrolysable tannins had greater ability to decrease CH_4 production and CH_4 production per unit organic matter digested than condensed tannins. The condensed tannins appear to decrease CH_4 more through a reduction in fibre digestion (indirect effect), while hydrolysable tannins appear to act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect).

The saponin-containing plants did not produce substantial reductions in CH_4 production but showed the potential to partition higher proportions of the substrate to production of microbial mass. The saponins tested possessed anti-protozoal activity but did not result in CH_4 inhibition suggesting that the uni-directional relationship between protozoal numbers and methanogenesis, as affected by

Figure 2. Effect of saponin-rich fractions on percent changes in microbial population.

saponins, is not obligatory. However, the inhibition of methanogen population led to increases in fibre-degrading bacterial groups.

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Evaluation of *Leucaena leucocephala* and *Ziziphus mauritiana* as Sources of Tannins and their Interference with Nitrogen Utilisation in Goats

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ABSTRACT

Studies were undertaken to identify the roles of tannins in *Leucaena leucocephala* and *Ziziphus mauritiana* on ruminal degradability of sesame meal crude protein (CP) using nylon bags and on nutrient digestibility of nitrogen utilisation in goats. Four diets were used in an *in situ* study using a fistulated bull: S (sesame meal), S+L$_1$ (sesame meal and *Leucaena leucocephala* [25% of the diet]), S+L$_2$ (sesame meal and *Leucaena leucocephala* [50% of the diet]) and S+Z (sesame meal and *Ziziphus mauritiana* [50% of the diet]). Crude protein disappearance of S+L$_1$, S+L$_2$ and S+Z were significantly lower (P < 0.001) than that of S, indicating that supplementation with *Leucaena leucocephala* and *Ziziphus mauritiana* lowered degradation of sesame meal protein, thereby saving more protein. A digestion trial was carried out using four indigenous male goats allocated randomly to four dietary treatments using a 4 × 4 Latin Square design: RS (chopped rice straw and sesame meal), RSL$_1$ (chopped rice straw, sesame meal and *Leucaena leucocephala* at 25% of ration), RSL$_2$ (chopped rice straw, sesame meal and *Leucaena leucocephala* at 50% of ration) and RSZ (chopped rice straw, sesame meal and *Ziziphus mauritiana* at 50% of ration). Dry matter (DM) and organic matter (OM) intakes of all diets were relatively similar, but CP intakes of RSL$_2$, RSL$_1$ and RS were significantly higher (P < 0.01) than that of RSZ. Digestibilities of DM, OM, CP, neutral detergent fibre (NDF) and acid detergent fibre (ADF) for RSZ were significantly lower (P < 0.01) than those of other treatments. The proportion of faecal nitrogen:total nitrogen intake (Nf/Ni, percentages) for RSZ was significantly higher (P < 0.01) than that of other diets while the proportion of nitrogen retention:total nitrogen intake (Nr/Ni, percentages) for RSL$_1$ tended to be higher compared with RS, RSL$_2$ and RSZ. Supplementing the ration with 25% *Leucaena leucocephala* tended to promote nitrogen retention. The results suggest that tannins in *Leucaena leucocephala* interfere with protein degradation in the rumen and improve nitrogen retention.

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INTRODUCTION

Rice straw is a major feed source for ruminants in many tropical countries including Myanmar (Clark, 1982), especially during dry seasons. Like other fibrous residues, it is a poor quality feed but its nutritional limitations may be overcome by supplementation with concentrates, urea or green forage (Preston and Leng, 1984). Supplementation of rice straw with concentrate improves its utilisation (Trung et al., 1988). Supplementation using by-products may increase intake and/or digestion and/or utilisation of the basal diet by improving the microbial activity required to optimise rumen digestion when ammonia nitrogen in the rumen is adequate (Tin Ngwe et al., 1993).

In Myanmar, sesame meal is one of the common feed supplements for draught cattle and cross-bred dairy cows fed rice straw. However, since sesame meal is highly degradable (88.7%) in the rumen (Sampath and Sivaraman, 1987) several processing treatments (heat, tannins, formaldehyde, etc.) have been used to increase the proportion of dietary protein which is not degraded in the rumen (Chalupa, 1975). While protection of highly degradable feed protein by heat and formaldehyde treatment have already been reported, little information is available about the effect of including the tannins in tree foliages on protein protection.

Conventionally, tree foliages have been fed together with agricultural by-products (mainly crop-residues containing low levels of nitrogen) to enhance rumen microbial fermentation and hence animal productivity (Ni Ni Maw et al., 2002). Tanniferous trees and shrubs are important in animal production because they can provide significant protein supplements (Makkar, 1999). Forages containing tannins include *Leucaena leucocephala, Ziziphus mauritiana, Albizia chinensis, Manihot esculenta,* and *Terminalia oblongata* (Kumar, 1992), and tree legume forages offer a cheap alternative to concentrate diets in ruminant nutrition (Abdulrazak and Ondiek, 1998).

Among tanniferous trees and shrubs, *Leucaena leucocephala* and *Ziziphus mauritiana* are common feedstuffs for goats in Myanmar. *Leucaena leucocephala* is now widespread through most tropical and sub-tropical regions of the world and provides an important source of feed for ruminant livestock (Norton et al., 1994). It is a high quality fodder tree (Liu and Wang, 1998) with a crude protein content of 28.8% in Myanmar, and therefore with considerable potential to replace commercial protein feed resources in rations or be used as a supplementary feed (Abdulrazak and Ondiek, 1998).

Tannins are generally regarded as inhibitory to the growth of microorganisms but the mechanisms involved are poorly understood. Petroleum ether, chloroform, methanol, etc., are used to extract tan-

nins sequentially from plant materials (Smith et al., 2001). However, detannification methods are very expensive and are not applicable in developing countries including Myanmar. Since tannins are widely distributed in tropical feedstuffs, if tree forages containing tannins were used to reduce protein degradability, savings on costs of providing dietary protein for ruminant production systems in developing countries may be expected.

Against this background, an experiment was undertaken to investigate the effect of tannins in *Leucaena leucocephala* and *Ziziphus mauritiana* on protein degradation of sesame meal using the nylon bag method in a fistulated bull. Another study examined the effect of *Leucaena leucocephala* and *Ziziphus mauritiana* on nutrient digestibility and nitrogen utilisation in goats.

MATERIALS AND METHODS

Experiment 1

Animals, Feed and Experimental Design
A fistulated bull (270 kg body weight [BWt]) was used to investigate the effect of *Leucaena leucocephala* and *Ziziphus mauritiana*, as sources of tannin on protein degradation. Four diets were used:
• Sesame meal (S)
• Sesame meal + *Leucaena leucocephala* 25% of the diet (S+L$_1$)
• Sesame meal + *Leucaena leucocephala* 50% of the diet (S+L$_2$)
• Sesame meal + *Ziziphus mauritiana* 50% of the diet (S+Z)

Before the study commenced a maintenance ration containing rice straw (4.75 kg), rice bran (220 g) and sesame meal (440 g) was fed 14 d. The experimental period for each diet lasted for 2 d. The experimental period lasted for 8 d.

Measurements
Dry matter, OM and protein degradation of each diet was measured by the nylon bag method (Orskov and McDonald, 1979). The sesame meal, *Leucaena leucocephala* and *Ziziphus mauritiana* were ground to pass through a 2 mm sieve. (Before incubation, the bags were dried in a hot air oven at 100 °C for 4 h to a constant weight). Bags (13.5 cm × 8.5 cm; pore size 50 µm) were used in this study, with eight incubation times for each diet, and three nylon bags being introduced into the rumen for each incubation time. Thus, twenty four bags were required to complete incubation of each diet. About 5 g of ground sample were weighed into the bag which was closed with a plastic tie and tied with plastic string. The bags were then suspended in the rumen by tying the string to a bamboo stick placed outside the cannula. Bags were incubated in the rumen for 0.5, 1, 3, 6, 12, 18, 24 or 48 h., withdrawn and washed immediately with cold water for about 1 h under running tap water while rubbing gently between the thumb and fingers until the water was clear. They were then dried under sunlight for 5 h., and finally to constant weight at 60 °C for 48 h in a hot air oven. After spreading on a table and allowed to equilibrate with the room temperature for 48 h the bags were weighed. Suitable amounts of residues were used to analyse for DM constituents.

Chemical Analysis
Dried residues were analysed for DM and OM as described by AOAC (1970). Nitrogen was determined using the Kjeldahl method (Foss 2020 digestor and Foss 2100 Kjeltec distillation unit), and CP calculated as 6.25×N (AOAC, 1970). All chemical analyses were carried out at the laboratory of Department of Physiology and Biochemistry, University of Veterinary Science, Yezin.

Statistical Analysis
The experimental results were subjected to one-way analysis of variance using ANOVA.

Experiment 2

Experimental Animals, their Management and Experimental Design
Four indigenous male goats aged about 5–7 months and BWt ranging from 19–29 kg were used to evaluate the effect of four dietary treatments on nutrient digestibility and nitrogen utilisation. Before starting the experiment, the goats were dewormed with Ivomec. During the study period, the goats were housed in individual metabolic stalls made of wood and an iron sieve and subjected to similar management practices. The feeding was done once daily at 09:00 h and the animals were given free access to water.

The experimental period for each dietary regime lasted 17 d. The goats were adapted to the test diet for the first 14 d and on the last 3 d of the experimental period samples of faeces and urine were collected from each goat for determination of nitrogen retention. The goats were randomly allocated to four dietary treatments using 4×4 Latin Square Design (**Table 1**). The dietary treatments were as follows:
• Chopped rice straw and sesame meal (RS);
• Chopped rice straw, sesame meal and *Leucaena leucocephala* 25% of ration (RSL$_1$);
• Chopped rice straw, sesame meal and *Leucaena leucocephala* 50% of ration (RSL$_2$);
• Chopped rice straw, sesame meal and *Ziziphus mauritiana* 50% of ration (RSZ)

All dietary treatments were adjusted to be isonitrogenous at the feeding level by calculating the crude protein content of each feedstuff contained in the diet. Dietary treatments were adjusted weekly by the supplement to levels of CP not less than 18% of the total diet.

Leucaena leucocephala and *Ziziphus mauritiana* (leaves and petioles) were harvested from the mature parts of the plant, collected, air dried and stored. The rice straws were also sun dried and chopped. Each diet was fed to goats at the level of 3.5% of BWt (as-fed basis). All feedstuffs used in the experiment were maintained as much as possible at a uniform composition throughout the trial period.

Amounts of all feedstuffs fed and refused were recorded daily for calculating intake levels. At the beginning of each period, animals were weighed before the morning feeding.

Measurements
Digestion trials were carried out by the conventional collection method. During collection periods samples of rice straw offered and refused and of sesame meal, *Leucaena leucocephala* and *Ziziphus mauritiana* were collected daily for chemical analysis. Residues were removed, weighed and sampled before the morning feeding.

Faeces voided and urine excreted were recorded daily during collection periods. The faeces from each goat were also weighed, sampled and put into a plastic bottle. After three consecutive d, 15% of faecal samples were dried under sunlight until constant weight was obtained. Urine was also measured, 10% sampled and stored in a refrigerator before chemical analysis. Five mL of 15% sulphuric acid was added to 200 g faecal samples before drying under sunlight and also added to the urine bucket before collection as preservative.

Table 1. Chemical composition (%) of diets incubated in the rumen of a fistulated bull.

Description	S	S+L$_1$	S+L$_2$	S+Z
DM[1]	91.8	92.2	92.0	92.3
OM	86.8	87.4	89.2	90.1
CP	40.8	34.4	33.4	25.7

S = sesame meal; S+L$_1$ = sesame meal + *Leucaena leucocephala* at 25% of diet; S+L$_2$ = sesame meal + *Leucaena leucocephala* at 50% of diet; S+Z = sesame meal + *Ziziphus mauritiana* at 50% of diet; [1] All values except DM are on DM basis.
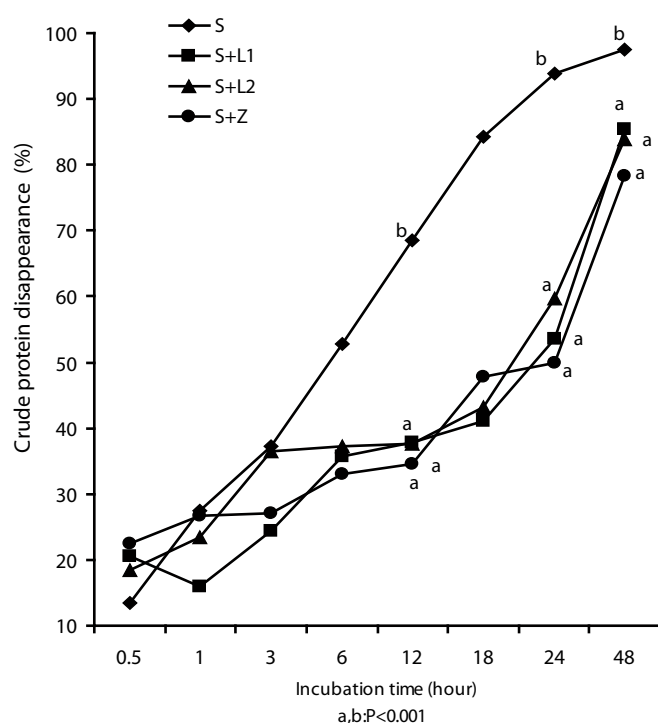
Figure 1. Disappearance of crude protein (%) of sesame meal supplemented with *Leucaena leucocephala* and *Ziziphus mauritiana* in the rumen of a fistulated bull

Chemical analysis
Ground samples of feed offered and of orts and faeces were analysed for dry matter (DM) and organic matter (OM) as described by AOAC (1970), and for neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and acid detergent insoluble nitrogen (ADIN) by the methods of Goering and Van Soest (1970). Faeces and urine were analysed for nitrogen using the Kjeldahl method (Foss 2020 digestor and Foss 2100 Kjeltec distillation unit) and crude protein (CP) was calculated as 6.25×N (AOAC, 1970). Estimates of tannin in *Leucaena leucocephala* and *Ziziphus mauritiana* were made by the sequential extraction of *Leucaena leucocephala* and *Ziziphus mauritiana* with acid detergent followed by neutral detergent.

Statistical analysis
Data were subjected to statistical analysis of ANOVA using Latin Square Design and the significance of differences between treatment means was compared by Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Experiment 1

Chemical Composition and CP Disappearance
Table 1 and **Figure 1** show the chemical composition of the diets and disappearance of CP from them. Dry matter and OM levels were similar in all diets, but CP content was highest in sesame meal and lowest where sesame meal was supplemented with *Ziziphus mauritiana* at 50% of in the diet.

The CP disappearances of all diets were relatively similar at 3 h incubation, but CP disappearances of S+L$_1$, S+L$_2$ and S+Z were lower than that of S at all times thereafter, significantly so (P < 0.001) after 12 h, 24 h and 48 h of incubation. This would indicate that supplementation with *Leucaena leucocephala* and *Ziziphus mauritiana* lowered the degradation of the sesame meal protein, and that this might be due to the presence of tannins in these foliages. This is consistent with the observation of Khin San Mu (2002) who reported that CP disappearance was reduced by incubating a concentrate with tamarind seed husk as a source of tannins to growing female calves. Also, Sampath and Sivaraman (1987) showed that the disappearances of DM and CP were reduced by incubating heat-treated sesame meal in the rumen, and Chalupa (1975) reported that several processing treatments (heat, tannin, formaldehyde, etc.) increased the proportion of dietary protein which is not degraded in the rumen.

Degradation Constants
The degradation constants of DM, OM and CP for S, S+L$_1$, S+L$_2$ and S+ Z are given in **Table 2**. Degradation rates (c) of DM, OM and CP for all diets were relatively similar but the values of b and a+b for S were found to be higher than those of S+L$_1$, S+L$_2$ and S+Z. The higher degradation constants of S would also indicate that tannins in these tree foliages protect the protein from degradation. The lower CP disappearance and degradation constants of the treatments that included *Leucaena leucocephala* and *Ziziphus mauritiana* suggest again that tannins contained in these tree foliages interfered with protein degradation in the rumen of the fistulated bull.

Experiment 2

Chemical Composition of Feedstuffs
The chemical composition of the feedstuffs are presented in **Table 3** (all values except DM expressed on DM basis). The CP content of *Leucaena leucocephala* used in this experiment was 28.8% which was higher than reported earlier (Smith, 1992; Wheeler et al., 1994; Ni Ni Maw et al., 2002; Lwin Naing Oo, 2002; Met Aung, 2002; Moe Moe Khaing, 2003), but similar to values reported by others (Jones, 1979; Abdulrazak and Ondiek, 1998; Aregheore and Yahaya, 2001).

The CP content of *Ziziphus mauritiana* was 13.9% which was similar to the value of 14% reported by Nath et al. (1969) and lower than the 18.39% recorded by Ni Ni Maw et al. (2002). The NDF and ADF values of *Ziziphus mauritiana* used were 35.8% and 25%

Table 2. Degradation constants of diets in the rumen of a fistulated bull.

DM [1)]	Description			
	S	S+L$_1$	S+L$_2$	S+Z
a, %	5.0	16.0	12.0	2.0
b, %	88.0	54.0	60.0	56.0
c, h^{-1}	0.085	0.084	0.085	0.090
a+b, %	93.0	70.0	72.0	58.0
OM [1)]				
a, %	7.0	10.0	9.0	1.0
b, %	87.0	58.0	61.0	57.0
c, h^{-1}	0.088	0.088	0.090	0.088
a+b, %	94.0	68.0	70.0	58.0
CP [1)]				
a, %	5.0	1.0	9.0	10.0
b, %	93.0	63.0	58.0	51.0
c, h^{-1}	0.091	0.087	0.088	0.087
a+b, %	98.0	64.0	67.0	61.0

a — rapidly degradable fraction; b — slowly degradable fraction; a+b — potentially degradable fraction; c — rate of degradation; 1 as described in Table 1.

Exponential equation: $P=A+B\ (1-e^{-kdt})$.

Table 3. Chemical composition (%) of feedstuffs.

Description	Rice straw	Sesame meal	Leucaena leucocephala	Ziziphus mauritiana
DM	87.7	86.0	89.3	90.6
OM	81.4	84.8	91.0	92.2
CP	5.7	40.6	28.8	13.9
EE	1.7	10.5	8.2	4.2
NDF	68.3	17.4	22.7	35.8
ADF	41.2	9.6	13.7	25.0
ADL	—	—	3.7	8.3
Tannin	—	—	2.0	4.8
Silica	—	—	0.3	0.5

EE — ether extract; ADL— acid detergent lignin.

respectively, higher than the 30.0% and 19.78% reported by Ni Ni Maw et al. (2002).

The difference of 2.0% between ADF and NDF in the sequential analysis of *Leucaena leucocephala* for tannins was in agreement with 1.4 - 7.9% reported by Wheeler et al. (1994), while the difference of 4.8% between ADF and NDF in the sequential analysis of *Ziziphus mauritiana* for tannins agreed closely with the 5.3% reported by Bhatia et al. (1987).

The differences in chemical composition between *Leucaena leucocephala* and *Ziziphus mauritiana* used in this experiment and from other observations likely reflected differences between parts of the plants used, their maturity, soil, weather and environmental characteristics.

Digestibility coefficients

DM digestibility of RSL$_2$ was significantly higher (P < 0.01) than that of RS but CP digestibility was significantly lower (P < 0.01). This might be due to the greater amount of *Leucaena leucocephala* in the ration which resulted in an increased amount of the tannin. The OM digestibilities of RSL$_1$ and RSL$_2$ were not significantly different from that of RS (**Table 6**).

Although all dietary treatments were adjusted to be isonitrogenous at the feeding level, a significant decrease was observed in CP intake of the RSZ diet (**Table 5**). This was due to underestimation of the CP content of *Leucaena leucocephala* and overestimation of CP content of *Ziziphus mauritiana*. Therefore, it could be assumed that all nutrient digestibilities were significantly (P < 0.01) reduced (**Table 6**) due to decreased CP intake in RSZ diet (**Table 5**). It is generally agreed that intake and digestion by ruminants is limited when the roughage contains less than 7% CP (Doyle, 1987). However, the CP content of RSZ constituted 15.7% of the diet, well above the CP content that may have limited nutrient digestibility. Therefore, the reduced nutrient digestibility might be due to ADIN content in *Ziziphus mauritiana* (**Table 4**).

Table 4. Content of acid detergent insoluble nitrogen in *Leucaena leucocephala* **and** *Ziziphus mauritiana.*

Description	Leucaena leucocephala	Ziziphus mauritiana
Total N %	4.6	2.2
ADIN %	1.7	1.5
ADIN/total N, %	37.0	68.0

N: = nitrogen; ADIN: = acid detergent insoluble nitrogen.

Table 5. Nutrient intakes (g/kg $^{0.75}$/d).

Description[1)]	RS	RSL_1	RSL_2	RSZ
DM I	59.3	55.5	63.4	63.2
OMI	49.2	47.2	55.1	55.4
CPI	12.5	13.0	15.2	10.2

[1] DMI — dry matter intake; OMI — organic matter intake; CPI — crude protein intake.

Table 6. Digestibility of nutrients (%).

Description	RS	RSL_1	RSL_2	RSZ	SEM
DM digestibility	59.0[B]	61.7[Aa]	62.0[Aa]	55.8[C]	0.591
OM digestibility	66.6[Aa]	67.7[Aa]	66.8[Aa]	60.2[B]	0.533
CP digestibility	82.5[Aa]	80.5[ABa]	75.5[Bb]	57.9[C]	1.390
NDF digestibility	59.8[Aa]	55.3[ABb]	52.1[Bb]	42.3[C]	1.107
ADF digestibility	56.8[Aa]	47.9[ABb]	41.9[Bb]	26.1[C]	2.479

Significant differences between means over the whole experiment are indicated by dissimilar superscripts: [A,B,C]P < 0.01 and [a,b]P < 0.05.

Table 7. Nitrogen utilisation by goats fed different diets.

Description	RS	RSL_1	RSL_2	RSZ	SEM
Total NI, g/d	20.1[Aa]	20.5[Aa]	24.5[B]	16.6[C]	0.649
Faecal N, g/d	3.5[Bc]	4.0[Bc]	5.9[Ab]	6.9[Aa]	0.231
Urinary N, g/d	11.8[ABa]	11.0[ABa]	13.4[Aa]	6.5[Bb]	1.331
N retention, g/d	4.8	5.5	5.1	3.2	—
Nf/NI, %	17.5[Aa]	19.5[ABa]	24.5[Bb]	42.1[C]	1.327
Nu/NI, %	59.6	54.5	55.0	40.3	—
Nr/NI, %	22.9	26.0	20.5	17.6	—
Nf/DNI, %	21.3[Aa]	24.2[Aa]	32.7[Aa]	73.6[B]	3.622
Nu/DNI, %	72.0	68.0	73.0	70.3	—
Nr/DNI, %	28.0	32.0	27.0	29.7	—

NI: = nitrogen intake; Nf: = faecal nitrogen; Nu: = urinary nitrogen; Nr: = nitrogen retention; DNI: = digestible nitrogen intake.

Significant differences between treatment means over the whole experiment indicated by dissimilar superscript: [A,B,C]P < 0.01; [a,b,c]P < 0.05

The NDF and ADF digestibilities of RSL_2 and RSZ were significantly lower (P < 0.01) than that of RS. Likewise, NDF and ADF digestibilities for RSL_1 were significantly lower (P < 0.05) than for RS, which is in accord with the report of Reed et al. (1990) that tannins have a negative effect on fibre digestibility (**Table 6**).

Nitrogen utilisation

Total nitrogen intakes of RS, RSL_1, RSL_2 and RSZ were 20.1, 20.5, 24.5 and 16.6 g/d, respectively (**Table 7)**. Total nitrogen intake of RSL_2 was significantly higher (P < 0.01) than those of other diets while total nitrogen intake of RSZ was significantly lower (P <0.01) than those of other diets.

The proportion of faecal nitrogen to total nitrogen intake (Nf/NI, %) of RSZ was significantly higher (P < 0.01) than RSL_2, RSL_1 and RS. This might be due to the high content of acid detergent insoluble nitrogen (ADIN) in *Ziziphus mauritiana* (68% of total nitrogen) com-

pared with that of *Leucaena leucocephala* (37% of total nitrogen) (**Table 4**). This is in agreement with the report of Nakamura et al. (1994) who showed that ADIN and nitrogen digestibility were correlated (r^2 = 0.66) and that ADIN was completely indigestible leading to underestimation of nitrogen digestibility.

The proportions of urinary nitrogen to total nitrogen intake (Nu/NI, %) of RS, RSL_1, RSL_2 and RSZ were not significantly different (P > 0.05). The Nu/NI, % of RSZ (40.3%) was numerically lower than those of RSL_1, RSL_2 and RS (59.6, 54.5 and 55.0% respectively), which might be due to the high content of tannins in the RSZ diet. However, the proportion of nitrogen retention to nitrogen intake (Nr/NI, %) of RSZ tended to decrease compared with other treatments which might be explained by an inadequate ammonia nitrogen concentration in the rumen for nitrogen utilisation because of the higher content of ADIN in *Ziziphus mauritiana* (68% total nitro-

gen) (**Table 4**). This was confirmed by the higher faecal excretion of nitrogen in RSZ diet (**Table 7**).

The proportion of nitrogen retention to nitrogen intake (Nr/NI, %) of RSL_2 was numerically lower than that of RSL_1, although the amount of tannin included in the RSL_2 was doubkle that of RSL_1. This would indicate an excessive amount of *Leucaena leucocephala* in the ration of RSL_2.

Although NDF and ADF digestibilities of RSL_1 were significantly lower than for RS (P < 0.05), the proportion of nitrogen retained to total nitrogen intake (Nr/NI, %) with the RSL_1 diet tended to be higher compared with other diets. Moreover, OM digestibility (**Table 6**) and the nutritive value of RSL_1 diet were also higher (**Table 7**). This observation is in agreement Dutta et al. (1999) who reported that the intakes $(g/kgW^{0.75})$ of DCP, TDN and the nitrogen balances of goats were significantly higher (P < 0.05) when *Leucaena* was fed. Norton (1994) also reported that nitrogen balance was apparently improved in animals that are fed low levels of tannins, although digestibility of forage fibre may be lowered.

No significant difference was observed in Nu/DNI, % and Nr/DNI, % among treatments suggesting that post ruminal nitrogen metabolism in goats fed on all diets was relatively similar.

Bhatta et al. (2000) reported decreased nitrogen excretion in urine with subsequent increased nitrogen retention in crossbred dairy cows fed with tamarind (*Tamarindus indica*) seed husks. Similar findings have been reported by Karda et al. (1998) in sheep fed with *Leucaena leucocephala,* by Barry et al. (1986) in sheep fed with *Lotus* and Pritchard et al. (1992) in sheep fed with *Acacia*, by Tin Ngwe (2003) in sheep supplemented with lablab bean (*Dlichos lablab*) husk, and by Lwin Naing Oo (2002) in goats supplemented with *Leucaena leucocephala.* In all these cases, the higher nitrogen retention was attributed to the tannin content of these legumes causing a reduction in protein fermentation in the rumen and an improvement in the efficiency of nitrogen utilisation.

Although tannins are regarded as antinutritional factors, certain types of tannins at low concentration are known to alter rumen fermentation of carbohydrates and protein (Barry and Duncan, 1984) and microbial protein synthesis (Makkar et al. 1995) to the benefit of ruminants. *Leucaena leucocephala* fed at the level of 25% of the ration used in the present experiment may have played roles favourable for nitrogen utilisation, while the higher level of ADIN content in *Ziziphus mauritiana* might have been a drawback as a ruminant feed.

CONCLUSIONS

Compared with other diets, the proportion of nitrogen retained to total nitrogen intake tended to be higher when the diet was supplemented with 25% of *Leucaena leucocephala.* The level of 25% of *Leucaena leucocephala* in the ration was found to be a good supplement to rice straw in terms of promoting nitrogen utilisation without reducing DM and OM digestibilities and could therefore be used as a source of tannins for protecting protein provided as concentrate from degradation in the rumen.

Ziziphus mauritiana reduced both fibre and protein digestibility because of its higher ADIN content and might therefore not be so suitable as a feed for goats.

ACKNOWLEDGEMENTS

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Application of Near Infrared Spectroscopy to Improve Animal Production in Developing Countries

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ABSTRACT

Near infrared (NIR) spectroscopy is an analytical technique measuring light absorption in the 780–2 500 nm region which is closely related to important chemical bonds (OH, NH and CH). NIR can be used to measure many nutritionally important constituents of concentrate and forage feeds, and from NIR spectra of faeces (i.e. dung) many constituents of the diet of grazing livestock. NIR depends on the development, in representative sets of samples, of mathematical relationships (calibration equations) between spectra and the constituents or attributes measured by conventional chemistry, and then application of these calibrations to estimate the constituents in unknown samples. These NIR calibration equations tend to be specific to the circumstances of the data used for their development. Application of NIR technology to livestock nutrition allows rapid, routine and economical analysis of feedstuffs and ingredients for compounded diets or supplements, thus improving stockfeed manufacture. Also NIR analysis of faeces allows estimation of the diet selected by grazing livestock and in small holder farming systems; this is not possible with any other technology. However, NIR instrumentation requires substantial capital investment, and considerable technical skills are required to develop and maintain calibration equations. Application of NIR technology allows established knowledge of the science of animal nutrition to be readily and objectively applied to improve productivity and cost-effectiveness of livestock production systems. Widespread use of this technology in developing countries would greatly improve quality control in manufacturing livestock feeds and application of existing nutritional knowledge to increase productivity and cost-effectiveness of livestock production.

Key words: *animal nutrition, ruminant nutrition, grazing animals, stockfeed.*

INTRODUCTION

Near infrared (NIR) spectroscopy is an analytical technique involving the measurement of absorption of electromagnetic radiation in the NIR region (780–2500 nm) of the spectrum of light (Osborne et al., 1993; Williams and Norris, 2001; Roberts et al., 2004a). An overview of the development and variety of NIR technology, and application in a wide range of industries and situations has been given by McClure (2003). Because absorption of NIR radiation is responsive to some chemical bonds (predominantly OH, NH and CH) the technique can be used to analyse many organic constituents of plant and animal tissues. During the last two decades NIR spectroscopy has developed for widespread, routine use in the food and agricultural industries in most developed countries, particularly for attributes of grains, forages, dairy products, and many other foods. Such measurements are commonly used for quality control, automated process control and valuation along marketing chains (Roberts et al., 2004a). It is also used extensively in the pharmaceutical, petroleum, textiles and other manufacturing industries and medical diagnostics (Flinn, 2007). This widespread development and application of NIR spectroscopy (NIRS) has been associated with improved sensitivity, ruggedness and reduced costs of instrumentation, and the parallel development of desktop computing essential for the spectral data analysis. NIR is only one of a suite of spectroscopy technologies developed in recent decades which may provide rapid and economical measurement in a wide variety of applications, but NIR has been most widely developed in the agricultural and other land-use sciences.

In the context of animal nutrition in developed countries, NIR spectroscopy is widely used for rapid and economical measurement of feedstuff ingredients and of forages for both monogastric and ruminant animals. A wide range of nutritionally important constituents (e.g. proteins, fibres, starches and sugars) and related functional properties (e.g. digestibility and voluntary intake by the animal) of feedstuffs and forages can be measured from their absorption characteristics (Givens and Deaville, 1999; Roberts et al., 2004b). It has been described as 'the most practicable and exciting analytical technique to hit the agricultural and food industries since Johann Kjeldahl introduced the Kjeldahl test' (Williams and Norris, 2001), and 'in the context of livestock industries in developed countries NIRS has revolutionised the analysis and nutritional evaluation of animal feeds by providing a rapid means of examination' (Givens and Deaville, 1999).

NIR analysis depends on the development of mathematical relationships (calibration equations) between absorbances at various wavelengths in the NIR region and composition of reference samples determined by conventional procedures such as wet chemistry. The NIR absorbance spectra of unknown samples are then used with these calibration equations to estimate constituents and functional properties. NIR spectroscopy allows rapid and economical analysis with minimal sample preparation and without the generation of wastes. Although conventional laboratories are still needed to devel-

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op and adapt general calibrations for local conditions and maintain NIR calibration equations, the number of samples requiring conventional analytical procedures can be drastically reduced and there is often opportunity to centralise laboratories. Furthermore some nutritional attributes cannot be routinely measured by any other procedure. Some classes of modern NIR instrumentation are designed to be rugged, reliable and portable, allowing use of the technology away from central laboratories and in the field, and with minimal training and technical support. For example such technology has been applied in the grains and horticulture industries using portable or handheld NIR spectrometers to provide real-time field measurement of maturity and quality of the crop.

In addition to direct measurement of feedstuffs, NIR spectroscopy of faeces (i.e. the dung) of ruminants has been developed to estimate many nutritional attributes of forage and forage-concentrate diets (Coates, 2004; Landau et al., 2006; Dixon and Coates, 2009). Prediction of diet attributes from the spectra of faeces is possible because there is sufficient information about the diet in the NIR spectra of faeces, despite the changes in chemical composition which occur during digestion in the gastrointestinal tract. However, because some constituents of the diet (e.g. the readily digested constituents) do not appear or appear in only low concentrations in faeces, the range of dietary attributes which can be measured from faecal spectra is more limited than the range which can be measured from the spectra of feeds. Nevertheless, faecal samples have the major advantages that they are usually easy to obtain and, because they represent the diet actually ingested, it is not necessary to obtain additional information

on the proportions of the various dietary components which are actually selected and ingested by the animal.

Apart from the measurement of the nutritional attributes of feedstuffs and the diet, NIR spectroscopy also can be used to measure many aspects of animal physiology and health such as reproductive status, reproductive and stress hormones, parasite burden, mastitis and a variety of metabolites. It can also evaluate many quality attributes of animal products such as milk and meat (Shepherd and Walsh, 2007; Dixon and Coates, 2009). However, NIR spectroscopy is generally not suitable for analysis of minerals, and concentrations of organic constituents generally have to be substantial (e.g. > 1%) for this technology to be applied.

ANALYSIS OF GRAINS, CONCENTRATE FEEDSTUFFS AND COMPOUNDED FEEDS FROM NIR SPECTRA OF FEED

NIR has been developed to measure many constituents and functional properties of numerous species and cultivars of grains. There is a vast literature, both public and in-house to laboratories, reporting such developments (e.g. Williams and Norris, 2001; Roberts et al., 2004b). In addition, numerous studies have developed quantitative NIR analyses for other materials commonly used as ingredients for compounded feedstuffs (e.g. by-products of food processing, protein meals) for both monogastric and ruminant livestock. A number of studies (e.g. de Boever et al., 1995; Aufrere et al., 1996; Mentink et al., 2006; **Table 1**) have shown that NIR calibrations of sufficient reliability and accuracy for most animal nutrition purposes can be

Table 1. Example of the calibration equation errors associated with NIRS analysis of compounded feedstuffs. The feedstuff mixtures were based on commonly available concentrates such as cereal grains, legume grains, protein meals and by-products of food processing (n = 433). (Perez-Martin et al., 2004).

Constituent	Actual content (lab reference value)			Predicted content		
	Mean	Minimum	Maximum	R^2	SECV	RPD
Moisture (g/kg)	103	60	136	0.85	5.4	2.6
Crude protein (g/kg)	184	119	333	0.98	5.0	7.2
Fat (g/kg)	54	16	168	0.95	4.7	4.6
Crude fibre (g/kg)	72	17	253	0.98	4.6	8.1
Ash (g/kg)	85	39	172	0.90	6.1	3.1

SECV — standard error of cross validation; RPD — ratio of the standard deviation of the actual reference data to the SECV (Williams 2001).

Table 2. Example of the calibration equation errors associated with NIR analysis of forage. Samples were obtained over four years from a heterogeneous and botanically complex semi-natural grassland in a temperate environment. The spectra of samples were measured using a scanning monochromator and calibrations calculated following a second derivative transformation of the absorbance data (Garcia-Ciudad et al., 1993).

Constituent	Actual content (lab reference values) Calibration (n = 97)			Predicted content Validation (n = 140)		
	Mean	Minimum	Maximum	R^2	SEP	RPD
Crude protein (g/kg)	99	43	181	0.90	5.8	3.1
Neutral detergent fibre(g/kg)	509	358	755	0.86	2.4	2.6
Acid detergent fibre (g/kg)	334	250	415	0.76	14.2	2.2
Lignin (g/kg)	47	25	99	0.88	4.5	3.3
Cellulose (g/kg)	284	212	345	0.74	12.7	2.1

SEP — standard error of performance; RPD — ratio of the standard deviation of the actual reference data to the SEP (Williams, 2001).

developed for the major nutritionally important constituents of mixed compounded feeds. Although NIR is generally considered not to be suitable for minerals or constituents present at less than about 10 g/kg, in the study summarised in **Table 1** calibration equations were developed for ingredients such as dicalcium phosphate, sodium bicarbonate and organic acids used as preservatives. A further observation in this latter study and others was that NIR spectra can be used to correctly identify many of the ingredients used to prepare the mixed feed. For example, a prohibited ingredient such as meat and bone meal could be easily and unequivocally identified.

ANALYSIS OF FORAGES FROM NIR SPECTRA

Numerous studies have examined NIRS for measurement of the composition and functional aspects of forages, and this application of the technology has been comprehensively reviewed (e.g. Givens and Deaville, 1999; Roberts et al., 2004b; Andres et al., 2005). Accurate and reliable calibration equations have been developed to predict composition including protein and related N compounds, various carbohydrates, components of fibre (crude fibre, neutral detergent fibre, acid detergent fibre, lignin), lipids, the rate and extent of rumen and entire tract digestion of N and carbohydrate fractions, digestibility of organic matter and dry matter, and antinutritional factors such as tannins and alkaloids. An example is given in **Table 2**. Predictions of digestibility of organic matter or dry matter (DM) have usually been more accurate and reliable with NIRS than with conventional laboratory approaches based on *in vitro* digestibility or via correlations with forage components such as acid detergent fibre or lignin (**Table 3**; Givens et al., 1992; de Boever et al., 1996; Andres et al., 2005). A number of studies have shown that NIR can be used to estimate the major botanical and morphological (e.g. leaf-stem) components of mixed forage material, albeit often with 'lumping' of minority components or of similar species (e.g. grasses).

Studies have examined the development of NIR calibrations to measure constituents and attributes of fresh forages and silages of high moisture content to avoid difficulties of loss of some constituents during drying and to enhance rapid measurement and field analysis. NIR analysis of high moisture materials is generally more difficult, and associated with much larger error, than measurement of dried and ground forages. This is a general constraint in NIR spectroscopy since water has strong absorptive properties which often obscure the spectral characteristics associated with other constituents. Never-

theless, calibrations have been developed with comparable prediction error to analysis of dried samples (Givens and Deaville, 1999; Park et al., 1999; Cozzolino et al., 2006).

Voluntary intake of forage by ruminants is another functional attribute of forages which in many studies has been predicted more satisfactorily from the NIR spectra of the forage than from any of the chemical constituents examined (Lippke and Barton, 1988; Givens and Deaville, 1999; Deaville and Flinn, 2000). As these authors discuss, this may well be because NIR measures numerous aspects of the chemical properties of the forage rather than any single constituent or group of constituents. The standard error of performance (SEP) of calibrations for voluntary intake of forage derived from the NIR spectra of the forage have usually been in the range 5–10 g DM/kg $W^{0.75}$.d. SEP values have usually been higher for high moisture materials such as silages than for dried forages such as hays.

ANALYSIS OF ANIMAL DIETS FROM NIR SPECTRA OF THE FAECES

Since it is usually vastly easier to obtain representative samples of faeces than of the diet ingested by grazing herbivores, the NIR spectra of faeces has been examined *in lieu* of the spectra of the diet to predict dietary attributes of grazing animals. Faecal NIR analysis has generally used the instrumentation, sample processing and chemometrics established for forage analysis. The NIR spectra of faeces have been used in two fundamental ways to provide information about the animal and its diet.

In the first approach NIR has been used to measure the concentrations of various constituents of faeces, the advantages of NIR (relative to conventional chemical analysis) being associated with cost, convenience and timeliness, and reduced handling of unpleasant samples. The concentrations of faecal constituents have then been used directly to provide information such as high moisture or fat concentrations indicating digestive abnormality, or have been used with assumptions from other experimentation (e.g. that digestion of condensed tannins or alkanes or the digesta marker polyethylene glycol was negligible, or that there are relationships between the concentration of N in faeces and diet digestibility) to provide information about the diet. Unfortunately the relationships between faecal N concentration and diet constituents can vary widely with the pasture system and between years (Corbett, 1978; Wang et al., 2009). These relationships may be applicable in some specific circumstances, but

Table 3. An example of the errors associated with estimation of organic matter digestibility *in vivo* (g/kg) of silages using NIR, or by *in vitro* disappearance using rumen fluid, pepsin-cellulase, or disappearance *in sacco* from nylon bags suspended in the rumen, or relationships with acid detergent or lignin content of the forage. Relationships were developed with one population (n = 122) and tested for an external population (n = 48) (after Barber et al., 1990).

Method	Regression between observed & predicted values (n = 122)		Regression between observed & predicted values in an external population (n = 48)			
	R^2	RSD	R^2	SEP	Slope	Bias
NIR calibration	0.85	-a	0.76	26	0.93	-8
In vitro rumen fluid	0.74	0.32	0.64	36	0.89	-19
In vitro pepsin-cellulase	0.55	0.42	0.40	47	0.71	23
In sacco disappearance	0.68	0.36	-	-	-	-
Acid detergent fibre	0.34	0.51	0.14	53	0.48	12
Lignin	0.52	0.44	0.20	51	0.52	-6

a — no residual standard deviation (RSD) value given, but standard error of calibration = 0.25.

OMD — organic matter digestibility; SEP — standard error of performance.
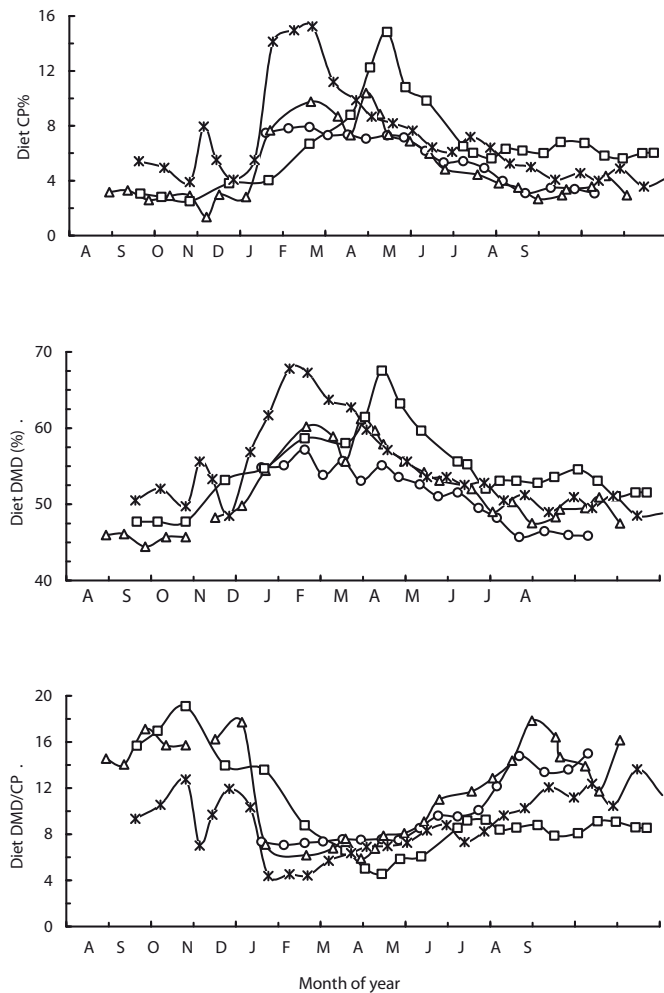
Figure 1. Dietary crude protein (CP; %) (A), dietary dry matter digestibility (DMD; %) (B), and the ratio of dietary DMD to CP (DMD/CP) in the diet (C) estimated from NIR spectra of faeces sampled at fortnightly intervals from four consecutive groups of *Bos indicus x Bos taurus* breeder cows grazing speargrass native pasture in a seasonally dry tropical environment at Millaroo, north Queensland, Australia during four annual cycles (2000-2003).
○ = Group 1; Δ = Group 2; □ = Group 3; x = Group 4 (after Dixon et al., 2007).

they are not generally useful. Other examples of the development of NIR to measure faecal constituents are the analysis of stress and reproductive hormones, of haemoglobin as a measure of some classes of parasite infection, and the $^{13}C:^{12}C$ ratio in faeces. This carbon isotope ratio is similar in faeces and in the diet. In tropical pastures the ratio in faeces allows estimation of the dietary proportions of grasses to non-grasses, the latter being comprised of dicotyledonous herbaceous plants and browses (Coates and Dixon, 2007 and 2008a). For a wide variety of tropical pastures the proportion of non-grass in the diet can be estimated from the NIR spectra of faeces with a standard error of performance of about five percentage units.

The second approach to application of NIR spectroscopy of faeces to predict diet has been to develop calibrations between the NIR spectra of faeces and the diet attributes of interest. In herbivores ingesting forage-based diets the NIR spectra of diet and faeces derived therefrom are similar, and sufficient spectral information is unchanged despite the processes of digestion to predict many dietary attributes (Brooks et al., 1984; Dixon and Coates, 2009). This is consistent with the observation that the faeces of herbivores consist principally of undigested plant material. Satisfactory calibrations can be developed to predict the crude protein and DM digestibility of forage and forage-concentrate diets with similar accuracy, reliability and limitations to NIR analysis of forages (Lyons and Stuth, 1992; Decruyenaere et al., 2009; Dixon and Coates, 2009). Limited studies indicate that it should be possible to develop general NIR calibrations for fibre fractions and condensed tannins in the diet. Prediction errors are likely to be greater for diet constituents of very high digestibility where there is little or negligible undigested residue in the faeces (e.g. soluble proteins or carbohydrates). Although most studies have been with cattle or goats, calibration equations have also been developed for non-ruminant herbivores (donkeys, kangaroos and ostriches) (Dixon and Coates, 2009). Also it is possible to predict the proportions of some plant species and groups of plant species (e.g. grasses), and the morphological components, at least in some circumstances.

Faecal NIR has been used to estimate the fluctuations in diet of grazing ruminants through annual cycles and also between years (**Figure 1**; Coates and Dixon, 2008b; Dixon, 2008). These data provide important information to enhance understanding of grazing livestock systems in the contexts of both the constraints of diet quality on the animal and the impact of the animal on the vegetation. For example, in the study shown in **Figure 1** animal responses to non-protein nitrogen supplementation would be expected when the ratio of DM digestibility to crude protein (DMD/CP) in the diet

Table 4. Examples of six studies with ruminants indicating the errors associated with calibration equations relating NIR spectra of faeces to the voluntary intake of diets of forage, or mixed forage and concentrates.

Animal species & class of diet	Units (VI/d)	n	Actual intake			Calibration		
			Mean	Min.	Max.	R^2	SECV	RPD
Cattle, pasture[A]	gDM/kgW	133	-	8	46	0.82	3.4	-
Cattle, forage [B]	gDM/W$^{0.75}$	139	101	58	157	0.98	6.8	4.6
Sheep, forage [C]	gOM/W$^{0.75}$	936	51	-	-	0.83	4.5	2.3
Cattle, forage [D]	gDM/kgW	472	16	4.2	28.6	0.85	1.9	2.4
Goats, mixed [E]	gDM	136	1031	552	1874	0.83	126	2.0
Goats, mixed [F]	gOM/W$^{0.75}$	305	28	-	-	0.90	5.4	1.9

References: [A]Coleman et al., 1995; [B]Decruyenaere et al., 2004; [C]Decruyenaere et al., 2009; [D]Coates, 2004; [E]Landau et al., 2004; [F]Landau et al., 2008.

VI = voluntary intake; DM = dry matter; OM = organic matter; W = animal live weight; SECV = standard error of cross validation; RPD = ratio of the standard deviation of the actual reference data to the SECV (Williams, 2001).
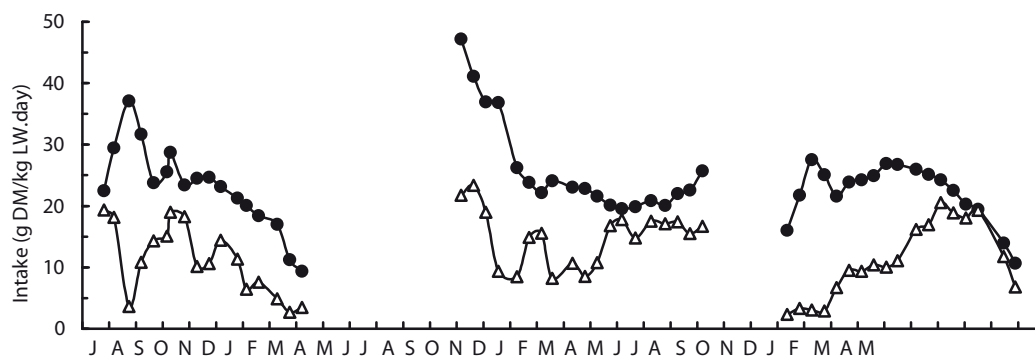
Figure 2. The estimated intakes of *Leucaena* dry matter (DM) (Δ) and total DM ( ) (g/kg LW.day) in three consecutive groups of *Bos indicus x Bos taurus* steers grazing in three consecutive years a *Leucaena*-grass pasture located in a subtropical environment at Gayndah, SE Queensland, Australia (Dixon and Coates, 2008a). Total DM intake was calculated from the metabolisable energy intake estimated to be required for the measured live weight change and the DM digestibility of the diet, while the *Leucaena* intake was calculated from the total DM intake and the proportion of *Leucaena* in the diet. The DM digestibility and the proportion of *Leucaena* in the diet were estimated from NIR spectra of faeces. The difference between the intakes of total DM and *Leucaena* DM was grass DM.

was > 8 (Dixon and Coates 2005). Past understanding of the quality of the diet selected and such responses by grazing animals has usually depended on procedures which are difficult and costly to implement and which involve large errors. The application of faecal NIR estimates of diet quality to improve management (e.g. nutrition, supplementation, weaning) of grazing cattle in a seasonally dry tropical environment has been discussed and provides a practical tool for improved productivity and cost-effectiveness of production (Dixon and Coates, 2005; Dixon et al., 2007).

A major constraint in the development of NIR spectroscopy of faeces to estimate the diet is that the calibration equations require faecal samples to be paired to samples of the diet ingested so that laboratory analysis of the diet can be conducted. This usually requires animals to be hand-fed in pens for intervals of 1–2 weeks. Such experimental procedures are costly and labour-intensive, and many forages selected by the grazing herbivore cannot be fed in this manner (Coates and Dixon 2009).

Voluntary intake of DM and liveweight change of ruminants can be predicted satisfactorily from the NIR spectra of faeces under some, but certainly not all, circumstances (**Table 4**; Coleman et al., 1995; Dixon and Coates, 2009). It seems likely that the NIR spectra of faeces are estimating primarily the variability of the diet, and prediction of voluntary intake is primarily a consequence of the associations between voluntary intake and characteristics of the diet such as DM digestibility and physical resistance to fragmentation and breakdown. This hypothesis is supported by the observations of Decruyenaere et al. (2009) that the same regions of the NIR spectra were predominant in the calibration equations for both DM digestibility and voluntary DM intake. The prediction errors for voluntary DM intake of forages are generally comparable with, or smaller than, the errors associated with prediction of voluntary DM intake from the NIR spectra of forages, or from conventional laboratory analysis of forage such as *in vitro* digestibility, neutral detergent fibre or acid detergent fibre. Faecal NIR predictions of voluntary DM intake would be expected to be an estimate of the potential intake in the class of animals utilised in the calibration data sets, as limited by forage characteristics, rather than necessarily actual intake which will be influenced by numerous aspects of the physiological state of the animal and the availability of the forage.

Since principles of energy metabolism in animals determine that there is a broad relationship between metabolisable energy intake (approximated by DM intake multiplied by DM digestibility) and liveweight change of an animal, calibrations for animal live weight change derived from the NIR spectra of faeces are to some extent comparable with calibrations for voluntary DM intake and diet DM digestibility. Satisfactory calibrations have been developed for animal live weight change when data were restricted to a specific class of animal (young healthy growing tropically-adapted cattle), although the error was quite large (standard error of cross-validation; SECV = 0.16 kg/d) (Dixon and Coates 2009). However, because both voluntary DM intake and live weight change are influenced by many animal factors (e.g. maturity, lactation, compensatory growth, parasites and disease), thermal environment and forage availability, it will be difficult to develop calibrations for either voluntary DM intake or live weight change applicable to a wide range of animal and pasture circumstances. In this regard, although the Coates (2004) live weight change calibrations predicted satisfactorily where cattle grazed pastures comparable with those in the calibration data set (Dixon et al., 2007; Coates and Dixon, 2008b; Dixon, 2008), large errors sometimes occurred for cattle grazing different pasture systems which were not represented in the calibration data set (Dixon, 2008, Dixon and Coates, 2008a), or for animals in different physiological states such as lactation, maturity or compensatory growth (Dixon et al., 2007; Dixon and Coates, 2008b). Nevertheless, as has often been observed during development of NIR calibrations, inclusion of some data representing a new pasture system has often radically improved the calibrations (Dixon and Coates, 2008 a and b). In conclusion, NIR spectra of faeces cannot directly predict voluntary intake where intake is modified by animal or environmental factors or where intake is constrained by the availability of the diet.

Despite these difficulties and constraints, faecal NIR technology has been applied satisfactorily to enhance knowledge of the nutrient intake and production of grazing cattle. For example, Dixon and Coates (2008a) used the actual live weight gain of young steers grazing *Leucaena* (a palatable tropical shrub) - grass pasture to calculate the metabolisable energy intake of the cattle, and faecal NIR predictions of diet DM digestibility and the proportion of *Leucaena* in the diet to estimate intakes of *Leucaena* and grass DM through three growing seasons (**Figure 2**). Furthermore, there was reasonable
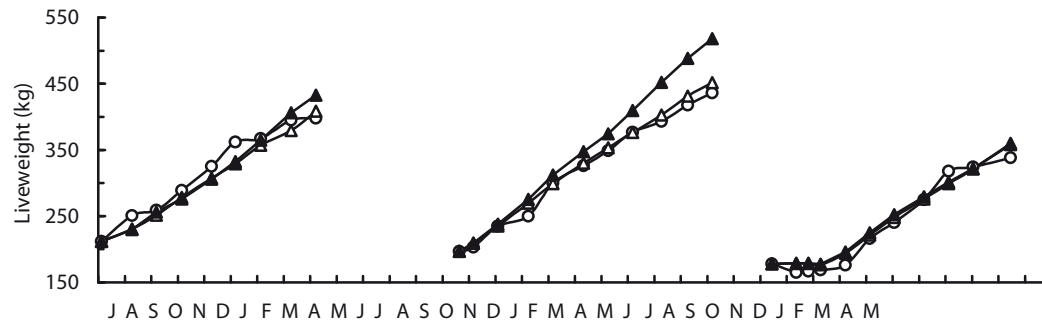
Figure 3. The actual measured live weight (○), and the cumulative live weight predicted from the NIR spectra of faeces calculated using two possible calibration equations (Δ, ▲) in three consecutive groups of *Bos indicus x Bos taurus* steers grazing a *Leucaena*-grass pasture located in a subtropical environment at Gayndah, SE Queensland, Australia (Dixon and Coates, 2008a).

agreement between the actual live weight gain of the cattle and the live weight gain predicted from faecal NIR calibrations provided that the calibrations were updated for the specific pasture system (**Figure 3**). There has also been reasonable agreement between observed and predicted live weight pathways of cattle grazing a number of other pastures in northern Australia (Coates and Dixon, 2008b; Dixon, 2008).

LIKELY ROLES OF NIR SPECTROSCOPY TO LIVESTOCK NUTRITION AND PRODUCTION SYSTEMS IN DEVELOPING COUNTRIES

In the stockfeed manufacturing industries, NIR spectroscopy can provide rapid and economical analysis of both concentrate and forage feedstuffs (including by-products) used as ingredients, and for quality control during manufacturing of products for both monogastric and ruminant animals. It thus allows quality control, and application of nutritional science in stockfeed manufacturing and thus animal production systems to an extent not previously practicable. In addition, NIR spectroscopy of faeces can provide routine estimation of the diet of ruminants in circumstances where this information is difficult or not possible to obtain using other technologies.

In developing countries NIR could be applied to understand the nutrition of grazing ruminants. It could also be readily applied to both ruminant and monogastric animals in small holder farming systems where diets will usually be derived from a diverse and changing array of local forages and feedstuffs, and where it is usually not possible to sample diet components adequately to estimate the diet consumed. Use of faecal NIR technology to better manage and improve livestock production systems in sub-Sahalan Africa and China have been demonstrated (Awuma, 2003).

In the context of developing countries NIR technology provides opportunities to:
- improve knowledge of the nutritional value, including the fluctuations through seasons and years, of regional and local resources used as livestock feedstuffs;
- apply the vast accumulated knowledge of livestock nutrition science to feeding livestock;
- use faecal analysis to simply and routinely monitor the diet and nutrition of livestock in local and regional production systems;
- improve nutritional management of livestock for improved productivity e.g. especially for milk production which is highly responsive to nutrition.

CONSTRAINTS TO IMPLEMENTATION IN DEVELOPING COUNTRIES

Numerous reviews such as those cited above have outlined and discussed the advantages of NIR technologies, and many of these advantages are as applicable to developing as to developed countries. In addition Shepherd and Walsh (2007) provide a comprehensive and thought-provoking overview and vision of the potential and possible role of such technologies for developing countries. Their focus, and many of the examples cited, relate to soil sciences and the African continent, but the vision is arguably equally applicable to other continents and other aspects of the agricultural and land-use sciences including livestock.

Major advantages of NIR technologies include:
- it provides rapid analysis, including with field-portable instruments in real-time;
- where appropriate calibrations equations are available the cost of analysis is much lower than that using conventional laboratory procedures;
- sample preparation is minimal (e.g. typically drying and grinding) or for some applications is not required (e.g. whole as-received grain or forage);
- no wastes are produced and no laboratory reagents are needed on a routine basis;
- routine analysis (e.g. as conducted in grain handling depots or on a factory floor in a country such as Australia) is possible by staff with minimal training or technical expertise (subject to type of instrument and application);
- some classes of modern instruments (e.g. diode array or [fourier transformed] FT-NIR) are rugged sealed units which should require little maintenance or repair. In some configurations such instruments are portable and suitable for field use. Data can usually be analysed by inbuilt electronics or down-loaded onto a laptop;
- a single NIR instrument and attached laptop can analyse a specific sample for a wide array of nutritional constituents or attributes thus reducing the need for a variety of laboratory instruments and lengthy procedures. Furthermore a single instrument (possible with several sampling attachments) can be used for a wide variety of feedstuffs and faecal samples, but also for a wide range of other agricultural materials and land-use related measurements (e.g. many foodstuffs, meat, milk, blood, soils).

The disadvantages of NIR technologies include:

- moderate to high capital cost of NIR instrumentation and software (e.g. US$15 000–150 000). This may be exacerbated by difficulties in obtaining technical support in developing countries;
- a high level of technical expertise, knowledge and skills are needed to develop or adapt existing calibration equations, for new and specific circumstances, and to trouble-shoot problems with development and maintenance of the instrument and data analysis. Considerable training and experience is usually required to become expert in chemometrics and the specialised software packages required;
- because calibration equations are usually quite specific for the product or material being measured they will usually need to be developed (or modified from elsewhere) for regional situations. This usually requires the analysis by both NIR and conventional chemistry of many hundreds of 'training' samples before analysis of unknown samples can commence. Although numerous laboratories and instrument companies have large data sets and calibration equations, these are usually regarded as intellectual property and often will not be made freely available;
- conventional laboratories still need to be maintained for high quality analysis of samples for development, and ongoing maintenance and refinement of calibration equations. Near infrared analysis can only be as accurate as the conventional laboratory analysis used to develop the calibration equations. Networks among laboratories are usually necessary to ensure quality control of conventional analysis and for conduct of ring tests. The advantage is that the number of analyses required in conventional laboratories should be greatly reduced;
- there will generally be a need for critical mass of scientific expertise across a range of disciplines to develop and maintain NIR spectroscopy groups.

An idealised design as a way forward to progress extensive development of infrared spectroscopy (both infrared and near infrared but where near infrared spectroscopy is likely to predominate) has been proposed by Shepherd and Walsh (2007). It involves developing regional centres of scientific and technical excellence to provide support for:
- high quality laboratory reference analysis;
- development of calibration databases and interpretation systems;
- upgrading of scientific and technical skills through training and education.

Key challenges for adoption of this design include:
- building human capacity in science and technology-based approaches, and developing understanding among both professional staff and clients in the industries and regions of interest on the role of these technologies. A particular need is to develop understanding and knowledge of spectroscopy among professionals as an essential basis for acceptance of the technology. Such knowledge is scarce in developed countries, far less in developing countries;
- development of rugged low cost infrared and near infrared spectroscopy instrumentation;
- development of decision support systems to interpret infrared spectroscopy data into management recommendations.

There are clear reasons that such centres of regional excellence in near infrared spectroscopy should not be limited to any one aspect of the technologies or to a single area or discipline of application. Many of the technical aspects of developing and improving instrumentation and use of data systems derived from this technology are likely to have much in common across disciplines and agricultural and land-use sciences.

CONCLUSIONS

NIR technology based on analysis of feedstuffs and faeces of livestock can rapidly and economically provide objective nutritional information on the diet of animals, their likely productivity, and of ingredients and processes for stockfeed manufacturing. It allows easy and comprehensive application of established nutritional science to the nutrition and management of livestock. This in turn improves the efficiency and productivity of livestock for food. Because a wide range of nutritional analyses can be conducted simultaneously with one instrument and a desktop computer, NIR spectroscopy can greatly reduce the capital investment, training and operational costs required for nutritional analysis and decision support. Nevertheless there are substantial obstacles to widespread implementation of NIR technologies in developing countries.

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Comparative Genomics for Prediction of the Relative Location of ESTs in the Goat Genome

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ABSTRACT

Worldwide the goat is an important agricultural species that is highly adaptable to many environmental conditions, and goat production is a rapidly growing industry within the USA. A better understanding of the goat genome could lead to new discoveries based on the genetic diversity and environmental adaptations important to ruminant health and production. An effort is underway to increase our understanding of the goat genome and develop a radiation hybrid (RH) map for stronger comparative genomic analyses. An embryo/uterine cDNA library was sequenced and about 12 800 expressed sequence tags (ESTs) added to the public database. In this study, comparative analyses among goat, sheep, and cattle maps were used to predict the location of the assembled EST contigs (n = 1 920) and singlets (n = 4 400) in the goat genome. Prediction of goat EST locations was determined through comparisons with the goat and sheep genetic maps using the bovine map as a backbone. Alignments of ESTs were predicted based on the relative location of mapped goat markers on the bovine sequence and refined by comparisons with the sheep maps. The predicted map attempts to localise the relative genomic position of the unique contigs and singlets developed from the available ESTs. Additionally, the degree of conservation among goat, cow, sheep, human, mouse, and rat genomes has been indicated and comparative maps generated. The predicted map will be a crucial resource for comparative genomic analyses and for the determination of EST and microsatellite markers during development of a goat RH map.

Key words: *goat genome, comparative genomics, radiation hybrid map, cDNA library, expressed sequence tags, markers.*

INTRODUCTION

Worldwide the goat is a primary source of milk, meat, and income to families and communities. The majority of the goats are found in developing countries, while in the USA the goat industry is relatively young and developing. Yet, the USA demand for goat products is greater than domestic production, which has led to an increase in the number of producers and animals over the last ten years. The majority of the world's goat producers are small, low-input farmers that need low-cost, effective mechanisms to address their individual disease and production needs. The discovery of the genes involved in phenotypic adaptations around the world can lead to the creation of inexpensive tests and when associated with local producer outreach programmes, can assist producers in the selection of animals that will be ideally suited to meet their environmental and production needs.

Comparative genomics is one of the more promising approaches for identifying the underlying causes for disease susceptibility and complex production traits. The worldwide phenotypic observations in the goat are an opportunity for scientists to utilise comparative genomics for identifying and understanding the underlying causes for a multitude of traits. However, the understanding of the goat at the genomic level is far behind other livestock species. A genetic map for the goat is available (Schibler et al., 1998), but it is somewhat dated and has a limited number of markers available for comparative mapping. With the current resources available, comparative analysis among the ruminant species is still challenging. However, development of a radiation hybrid (RH) map, which is available for sheep (Wu et al., 2007) and cattle (Womack et al., 1997), will allow for placement of a variety of marker types and comparisons with other species. The diversity of the goat populations and the development of a RH panel and map for the goat will allow scientists to use comparative analyses with the more developed bovine, sheep and human genomic resources to address the underlying genetic aspects of important traits. Additionally with the development of new technologies and the promise of reduced costs for genome sequencing looming in the future, the development of the RH map for the goat will provide a framework map which can be used to order the sequences.

The development of an RH map involves irradiating goat cells with 5 000 rad from a ^{60}Co source to randomly break the DNA. These irradiated cells are hybridised with hamster cells to form cells that contain both goat and hamster DNA segments. A panel of these hybrids is screened for the location of goat genomic markers. The relative frequency of markers in the panel can be used to determine their distance from each other and develop a RH map.

The development of the RH map for the goat will require the selection of markers throughout the genome to make the best use of the limited amount of DNA from the RH panel. The objective of this project was to develop a predicted gene map for the goat to assist in identifying genes and markers for developing a RH map.

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MATERIALS AND METHODS

Identification of EST Identities and Location

A total of 12 698 quality goat EST sequences were collected from the NCBI database. The sequences were previously clustered and assembled. From this process there were 1 921 contigs formed from 8 388 sequences, with 4 433 singles remaining (Sayre et al., 2006).

The 6 354 unique contigs and single sequences were subjected to a BLAST search of the NCBI RefSeq databases for sheep, cattle, human, mouse and rat. From the top hit for each EST sequence, the data collected was the percent of similarity of compared sequences, percent of the total EST sequence associated with the database sequence, the RefSeq accession number, and the description of the associated sequence from the database. The data were stored in Excel worksheets and Access databases.

The RefSeq accession data from the BLAST comparisons were used to withdraw information from the NCBI Gene database. The symbol, chromosomal location, and physical location data for each identified gene and species were extracted.

Development of the Predicted Goat Gene Map

To predict the location of the ESTs in the goat genome, a framework map based on the genetic maps was developed and followed with placement of the gene locations based on the cattle genomic map. The framework map was developed by localising the markers available on the goat genetic map with the sheep genetic and cattle genomic maps. The marker information was localised from the published genetic map for the goat (Schibler et al., 1998) and NCBI UniSTS for the physical location of the markers on the cattle genomic

Table 1. Total number of genes predicted and sequences conserved at 70% SI or greater in cattle, human, mouse and rat.

Chromosome	Genes	Cattle	Human	Mouse #a (%b)	Rat	Overall
1	98	67 (68)	37 (38)	23 (23)	23 (23)	23 (23)
2	141	89 (63)	54 (38)	33 (23)	31 (22)	29 (21)
3	196	144 (73)	93 (47)	59 (30)	54 (28)	55 (28)
4	86	58 (67)	28 (33)	15 (17)	13 (15)	12 (14)
5	190	126 (66)	77 (41)	55 (29)	55 (29)	51 (27)
6	74	56 (76)	33 (45)	23 (31)	20 (27)	19 (26)
7	220	160 (73)	92 (42)	63 (29)	63 (29)	61 (28)
8	96	74 (77)	45 (47)	32 (33)	31 (32)	31 (32)
9	63	43 (68)	19 (30)	17 (27)	13 (21)	15 (24)
10	133	85 (64)	45 (34)	31 (23)	27 (20)	29 (22)
11	154	109 (71)	64 (42)	45 (29)	39 (25)	45 (29)
12	58	28 (48)	14 (24)	8 (14)	8 (14)	8 (14)
13	130	91 (70)	58 (45)	38 (29)	35 (27)	36 (28)
14	62	40 (65)	25 (40)	13 (21)	11 (18)	12 (19)
15	105	79 (75)	52 (50)	41 (39)	36 (34)	37 (35)
16	70	41 (59)	20 (29)	14 (20)	13 (19)	16 (23)
17	101	75 (74)	47 (47)	36 (36)	37 (37)	34 (34)
18	185	121 (65)	76 (41)	60 (32)	55 (30)	55 (30)
19	227	145 (64)	90 (40)	69 (30)	66 (29)	69 (30)
20	38	25 (66)	13 (34)	10 (26)	8 (21)	7 (18)
21	86	56 (65)	32 (37)	22 (26)	20 (23)	22 (26)
22	102	72 (71)	48 (47)	31 (30)	33 (32)	28 (27)
23	94	63 (67)	36 (38)	19 (20)	16 (17)	20 (21)
24	41	33 (80)	20 (49)	14 (34)	12 (29)	13 (32)
25	137	92 (67)	49 (36)	34 (25)	31 (23)	35 (26)
26	57	42 (74)	23 (40)	18 (32)	16 (28)	17 (30)
27	33	23 (70)	12 (36)	7 (21)	8 (24)	8 (24)
28	45	32 (71)	23 (51)	15 (33)	12 (27)	12 (27)
29	91	66 (73)	46 (51)	33 (36)	30 (33)	31 (34)
X	77	45 (58)	19 (25)	14 (18)	15 (19)	20 (26)
Overall	3 190	2 180 (73)	1 290 (43)	892 (30)	831 (28)	850 (28)

a Number of the total genes from each chromosome conserved within various species.

b The percent of gene conservation within various species ([# of genes conserved / total # genes on the chromosome] × 100).

Figure 1. Predicted location of ESTs (green) on goat chromosomes 1–5 based on the location of known goat markers (blue).

Figure 2. Predicted location of ESTs (green) on goat chromosomes 6–10 based on the location of known goat markers (blue).
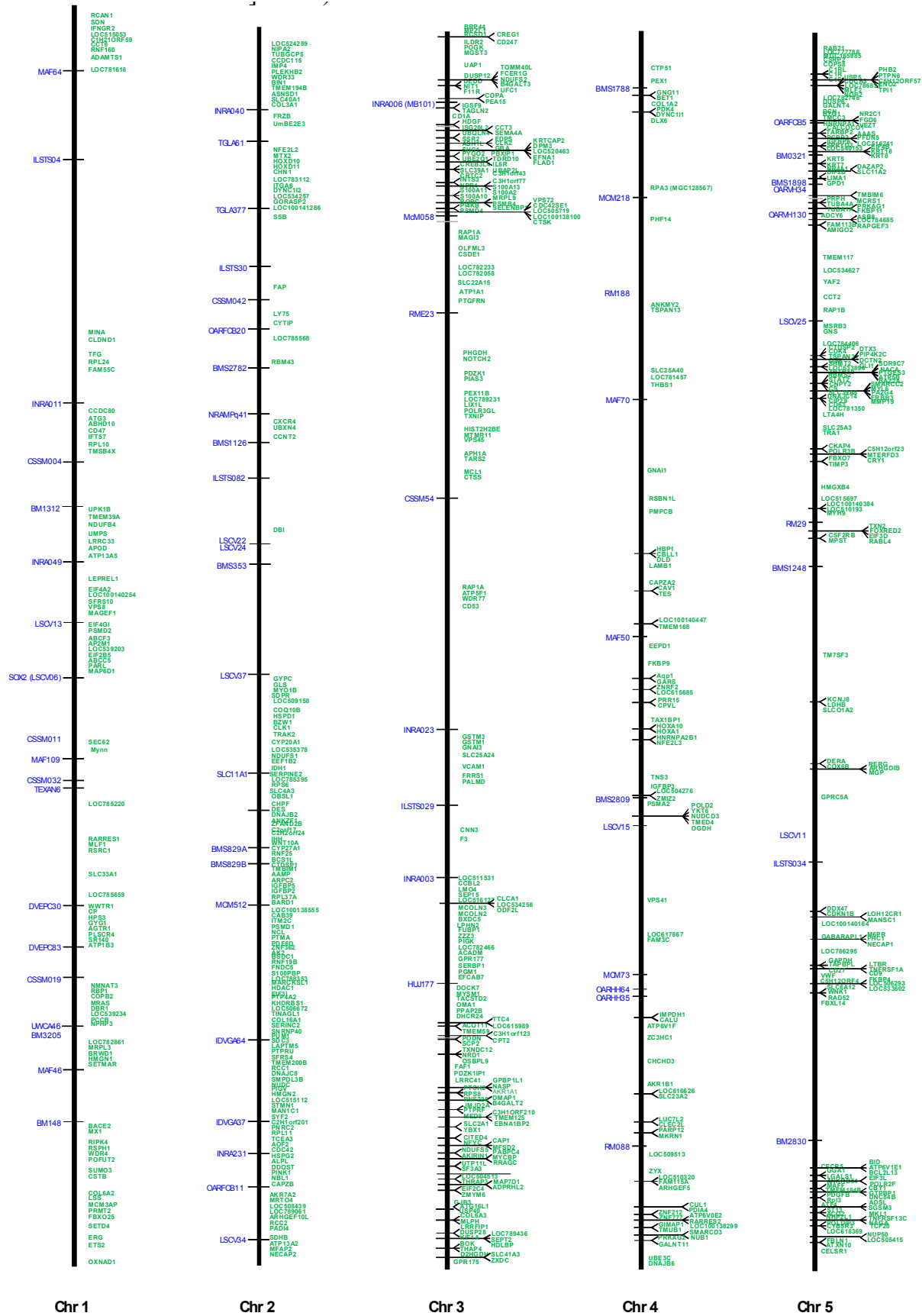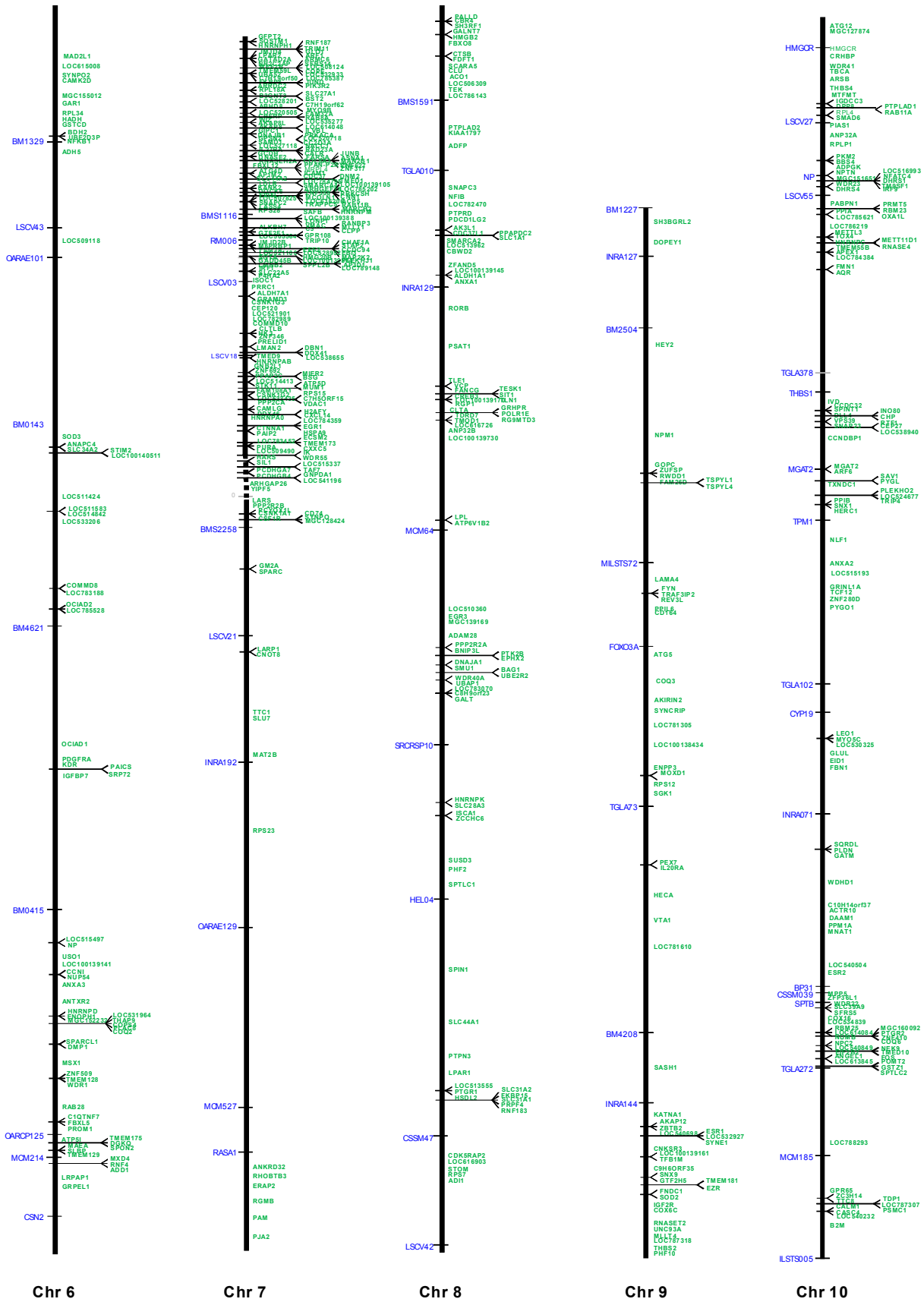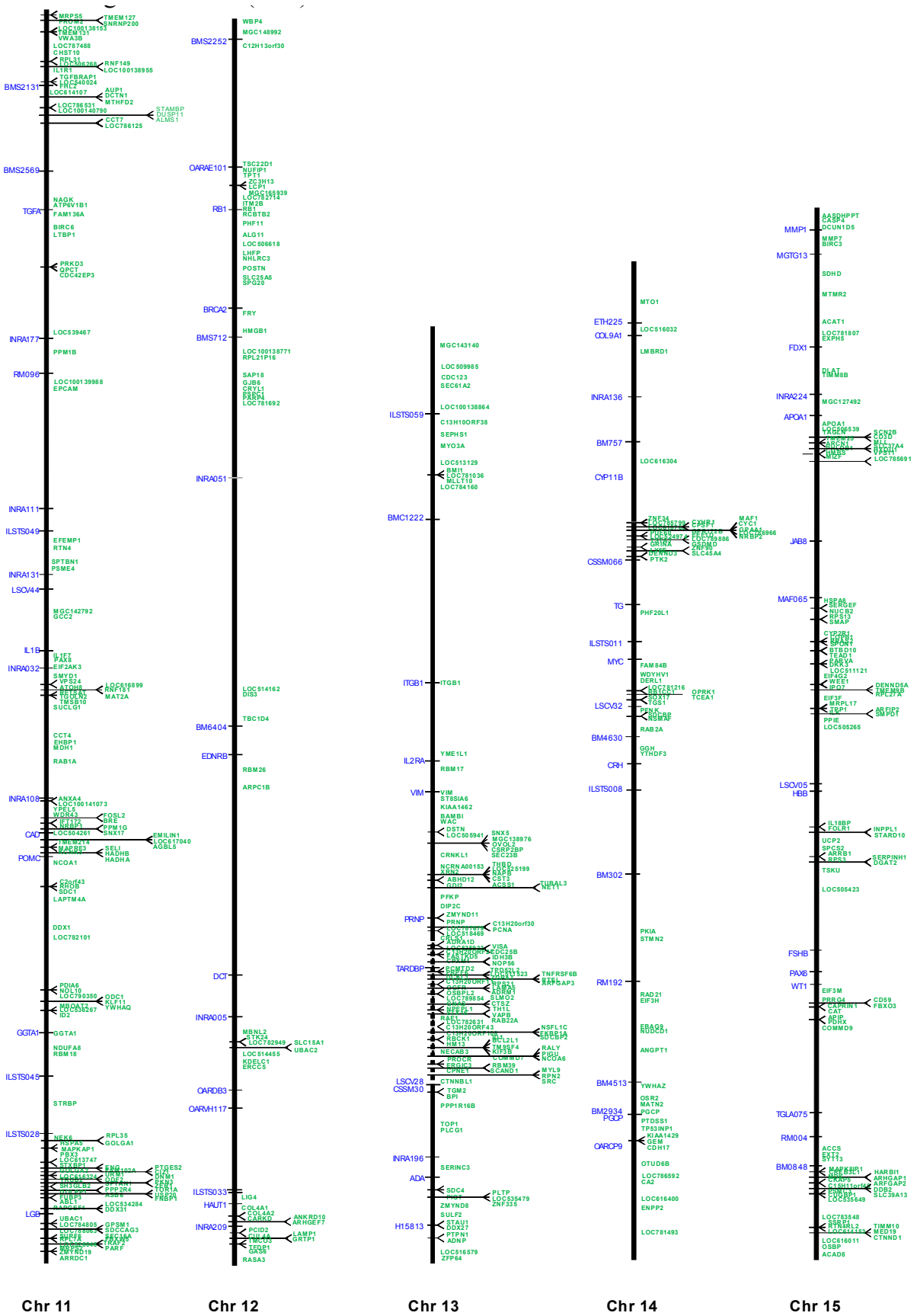
Figure 3. Predicted location of ESTs (green) on goat chromosomes 11–15 based on the location of known goat markers (blue).
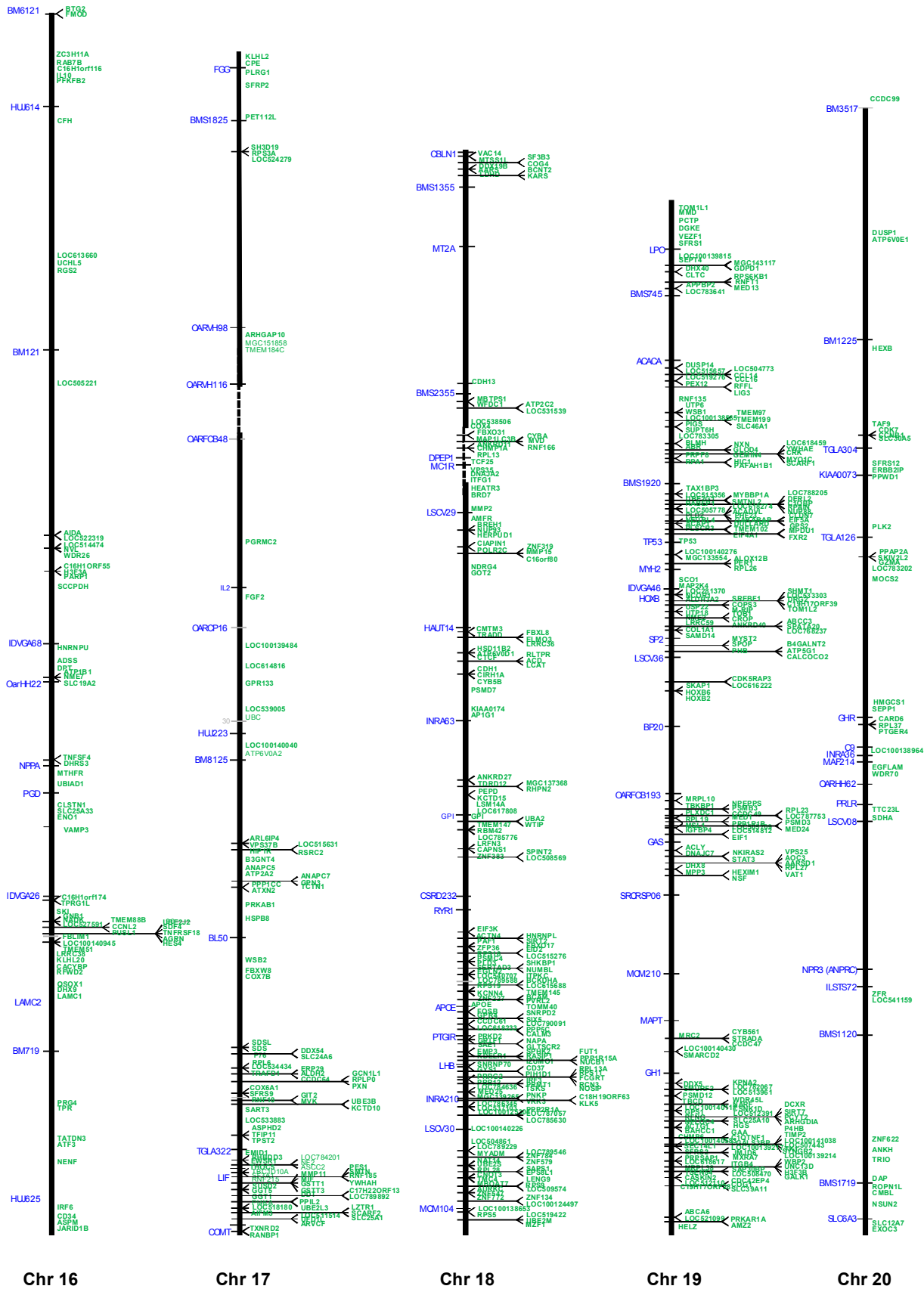
Figure 4. Predicted location of ESTs (green) on goat chromosomes 16–20 based on the location of known goat markers (blue).

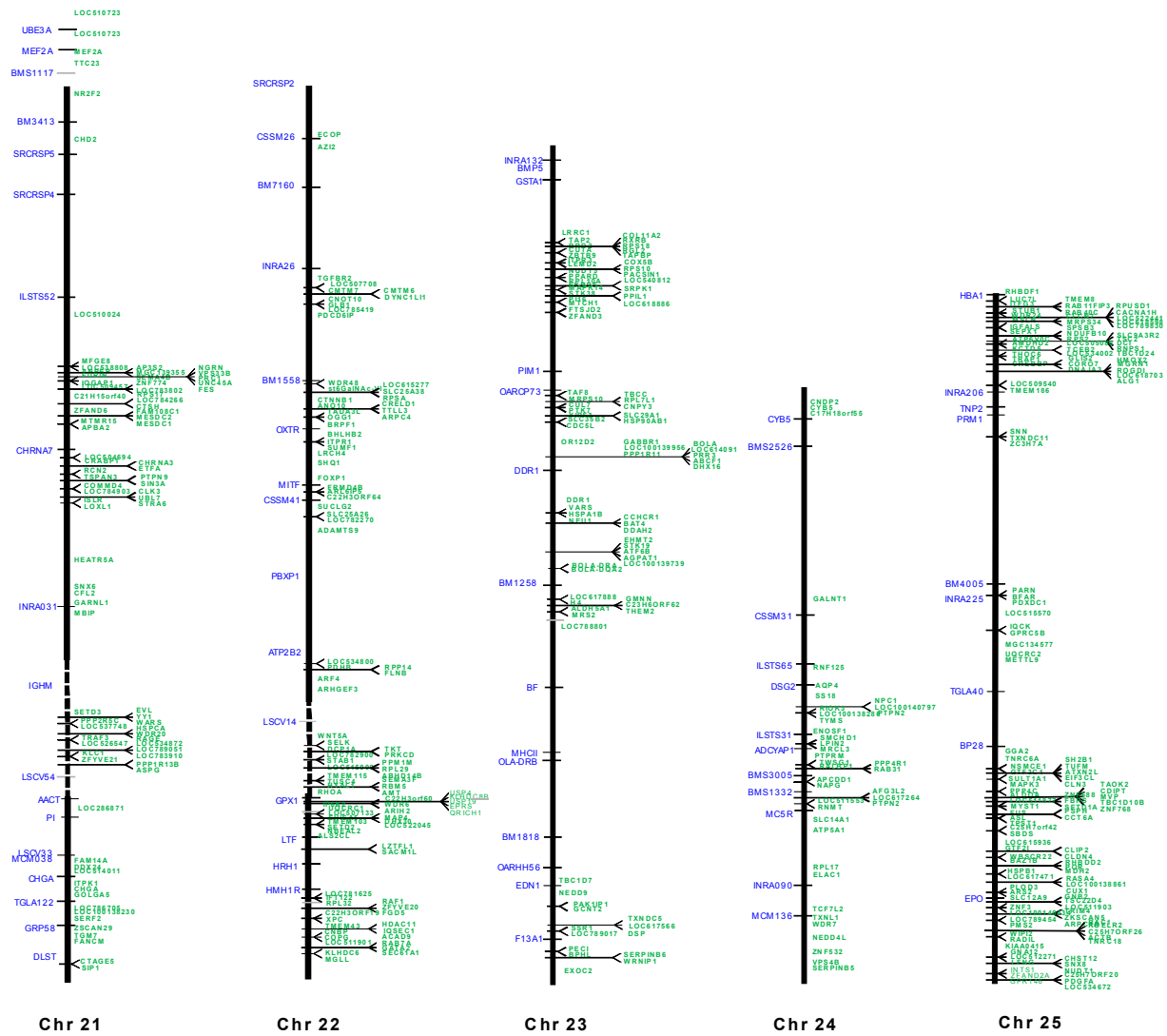**Chr 21** **Chr 22** **Chr 23** **Chr 24** **Chr 25**

Figure 5. Predicted location of ESTs (green) on goat chromosomes 21–25 based on the location of known goat markers (blue).

map. The placement of the genes on this framework began with the localisation of the gene in the cattle genomic map and properly ordering the genes relative to the locations of the genetic markers. Then based on the framework map, the locations of genes were predicted on the goat genome map.

To make comparisons of the species based on conservation, a sequence index (SI) was created that took into account the percent sequence similarity relative to the percent association. The index was SI = association percent x (similarity percent/100) x 100. Based on the SI, the conservation of the sequences was compared among the species and individual chromosomes.

RESULTS

We predicted the possible identity and genomic location of 3 190 EST sequences. The number of predicted EST sequences and the number of those sequences with greater than 70% conservation to the cattle, sheep, human, mouse and rat sequences is displayed in **Table 1**.

Conservation of gene sequences appeared to be evenly distributed across the chromosomes.

The predicted map includes 3 190 genes that have been distributed across the 29 autosomes and the X chromosomes in the goat (**Table 1**). The predicted locations can be visualised on the predicted map in **Figures 1–6**. The ESTs appear to be grouped to specific regions of the chromosomes while other regions have only few genes present. The gene symbols designating the EST identities can be found on the right side of the chromosome maps. The left side of the chromosome maps indicates the locations of the genetic and gene markers from the goat genetic map.

DISCUSSION

The map developed during this project will be used as a framework for the development of a RH map, which will be the first physical genomic map for the goat. The completion of the RH map will enable comparative analysis of marker association studies in the goat and the potential identification of genes related to particular phenotypes.
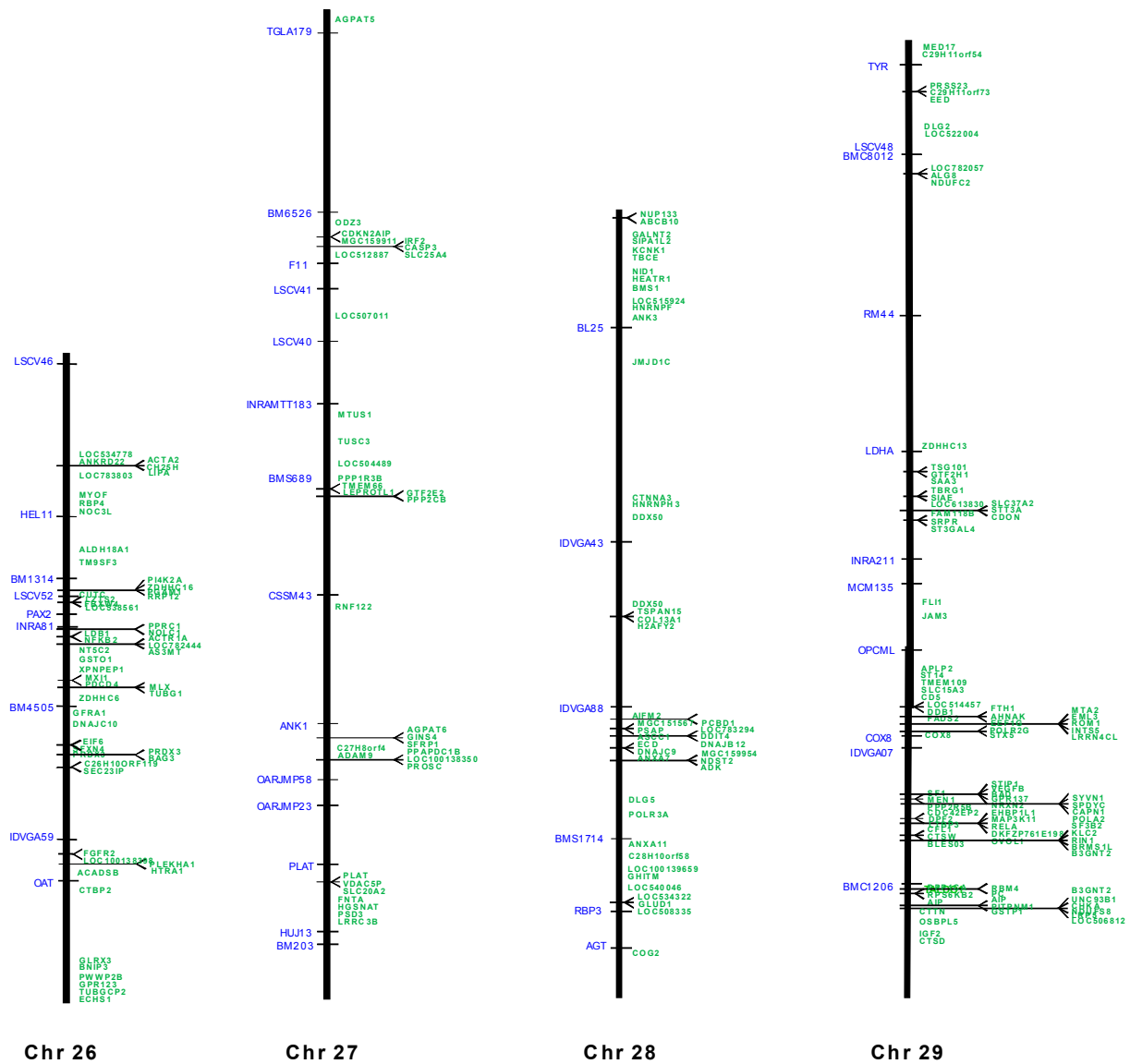
Figure 6. Predicted location of ESTs (green) on goat chromosomes 26–29 based on the location of known goat markers (blue).

Identification of the genes and markers can allow for local groups to assist goat producers in the selection of animals that will better survive and perform under their specific environmental conditions.

The predictive gene map was created to determine the potential location of the genes in the goat genome and relationships to the bovine sequence map. The sequencing of the goat genome would give researchers the best tools available for discovery of genes related to diseases and production issues. The development of this EST-based map will be useful in the assembly of the goat genome when sequencing commences. The EST-based map can provide the approximate locations of genes in the goat genome to assist in the ordering of sequence scaffolds during the genome assembly process. The production of a physical map for the goat would be the best comparative map. Until such time, comparative analyses among livestock species is one of the more promising approaches for identifying the underlying causes for disease susceptibility and production traits. The comparative analysis will be useful when identifying potential syntenic regions that may exist within quantitative trait loci (QTL) for similar traits.

CONCLUSIONS

This project identified the location of many of the goat ESTs and made comparisons with bovine, ovine, and human data. The development of this map is a valuable tool for development of physical and comparative genomic maps for the goat. Using comparative genomics, scientists can take advantage of the diversity of phenotypes found in the goat to address the underlying genetics of biomedical and production traits.

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Early Stirrings of Landscape Genomics: Awaiting Next-next Generation Sequencing Platforms before Take-off

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ABSTRACT

Landscape genomics is an emerging research field that bridges genetics and genomics to geo-environmental resources analysis. It aims to study genome/environment interaction to discover the genetic basis of adaptation by processing of many simultaneous DNA-environment association models, exploiting GIS (Geographical Information Systems) science and statistical methods. In this paper, we review the literature related to the recent history of this discipline, describing its application to livestock genetics, discussing its potential contribution to Farm Animal Genetic Resources (FAnGR) conservation and management, explaining its role in the analysis of the local adaptation of autochtonous breeds and showing how the upcoming next-next generation of DNA sequencing methods, in parallel with the availability of an increasing number of high quality environmental data sets, will allow a real take-off of this novel approach.

Key words: *landscape genomics, DNA sequencing, environment, geographical information systems, animal genetic resources.*

INTRODUCTION

Development of sustainable agriculture, including animal husbandry, based on adapted breeds is a priority for most countries in the world, and is of key importance to emerging countries in particular. The genetic basis and the level of adaptation of livestock breeds to their environment has to be investigated in order to reach a better understanding of the relationship between environment and adaptive fitness of livestock populations, in favour of production systems based on adapted local breeds. According to the Africa-based International Livestock Research Institute, landscape genomics seems to be a long term promising approach for understanding the genetic adaptation of livestock to the environment (ILRI, 2007).

LANDSCAPE GENOMICS

A wide definition considers this field as an "emerging area at the interface of natural resources management and the genome sciences" (Williams, 2004). Landscape genomics takes its roots in landscape genetics, a new approach described as the combination of landscape ecology and population genetics (Manel et al., 2003). Landscape genetics tries to facilitate our understanding of how geographical and environmental features structure genetic variation. Landscape genetics rapidly became a term used to describe all research about genetic data, exploiting their geographic dimension and spatial statistics (Storfer et al., 2007).

Implementation of the landscape genomics approach described by Luikart et al. (2003) was published by Joost et al. (2007) who described the detection of candidate loci for selection in insects (pine weevil) and in a livestock species (sheep). Population genomics, geo-environmental and statistical methods were combined to assess the level of association between specific genomic regions of living organisms and environmental factors. Association models (logistic regressions) between hundreds of environmental parameters and thousands of molecular markers from thousands of animals, were processed to identify genomic regions likely to be under natural selection.

Landscape genomics has also potential for supporting livestock conservation activities. The global purpose of this discipline is to uncover which genetic variations are likely to fit to environmental conditions, or to biogeographical regions worldwide. Indeed, autochthonous livestock are adapted to the landscape where they are bred. Since association models make it possible to go a step further to identify specific loci linked to environmental parameters compared with classical approaches (Joost et al., 2007), it is then possible to learn from the co-evolution of livestock and their production systems. In a subsequent step, acquired information can be used to better match different breeds with optimal production conditions (Long, 2008) and to produce, for example, maps of potential or optimal habitat (**Table 1**). Incidentally, the Host/Pathogen Interaction Programme funded by the Wellcome Trust and centred around bovine sleeping sickness in Africa initiated activities on landscape genomics with the goal of linking environmental factors, trypanosomiasis and cattle (http://www.genomics.liv.ac.uk/tryps/).

Landscape genomics is also of interest for fish biology (Nielsen and Hansen, 2008) and plant science (Holderegger et al., 2009), and the research strategy in the latter field uses of this promising approach. In a recent example from April 2009, the European Plant Science Organisation (EPSO) organised a workshop on landscape genomics (http://www.epsoweb.org/Catalog/epso_workshops/) to bring together ecologists, natural history collection experts and genomics specialists to discuss ideas and needs for future collaborative projects. This interdisciplinarity means that several research fields should contribute to future progress in landscape genomics.

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Table 1. Compulsory and optional components of livestock landscape genomics and expected outputs.

	Input	Output
Compulsory information to compute association models	Geo-environmental data (topographic and eco-climatic information, bio-physical information describing the production system e.g. type of pasture, soil, etc.) Geo-referenced genetic data (SNPs but also AFLPs, microsatellites, etc.)	Working hypotheses about the function of genome regions which are linked to the genetic markers Maps of potential habitat for breeds (Joost, 2008) Predictions about consequences of climate change (landscape change) Predictions about consequences of landscape change due to human activity (constructions)
Optional additional information on diseases and farming systems	Geo-referenced disease information Geo-referenced farming system data (socio-economic information on farms, management systems, goals, knowledge, resources, monitoring opportunities)	Working hypothesis on relationship among selected genomic regions, specific environmental configuration and disease occurrence

The first applications of landscape genomics were recently reported in wildlife (Joost et al., 2008), livestock (Joost et al., 2007; Pariset et al., 2009) and plants (Parisod and Joost, 2010). In livestock they are limited to studies carried out on sheep (Joost et al., 2007) and goat (Pariset et al., 2009), both being based on the use of Sam software (http://www.econogene.eu/software/sam/) as well as population genomics theoretical approaches for results validation (Foll and Gaggiotti, 2008). Far from fully exploiting the potential of landscape genomics, these reports are restricted to the simultaneous processing of approximately one hundred environmental parameters related to a small number of genetic markers (<1 000) produced from 2 000 animals at most. We will now discuss how these figures are expected to evolve over the short term, taking advantage of the availability of environmental data dealing with distinct characteristics, and considering the perspective offered by the next-next generation of molecular technologies.

ENVIRONMENTAL DATA

The environment in which livestock populations are reared directly affects animal health and production. Thus, geo-environmental data provide the framework for mapping and analysing disease occurrence, monitoring climate trends and characterising production environments in order to support evaluations and comparative analyses of livestock performance (FAO, 1998). Moreover, as mentioned above, this information is essential to understand the genetic basis of native livestock adaptation to their environments, and is therefore important for optimising the management of animal genetic resources (FAO, 2007).

Most of environmental global data sets are freely available from the Internet and can be used for a comparative description of production environments worldwide. The 'sustainable development' principle established during the United Nations Conference held in Rio de Janeiro (1992) promoted actions towards the collection of additional environmental data at different scales, and recommended that countries provide open access to the information for stakeholders and scientists involved in environmental decision-making processes (UN, 1992; Haklay, 2003). For instance, as a concrete consequence of this call, the Global Map project (http://www.globalmap.org/) proposes data sets comprising elevation, land cover, land use, and vegetation, as well as information on transportation, population and political boundaries. The project is supervised by the International Steering Committee for Global Mapping (Secretariat of ISCGM, 1998), with over 90 participating countries (Verdin and Jenson, 1996). Version 1.0 of the Global Map project consists of data contributed, updated and maintained by each country. The main international global environmental geodata sources are included into the Global Map project, and are available from the Secretariat for the ISCGM hosted by the Geographical Survey Institute of Japan.

In parallel to this action, several international or national agencies provided free access to geo-environmental data at different spatial resolutions and for different time periods. Among them, the most important are the European Environment Agency (http://www.eea.europa.eu/, EEA) and agencies in the USA like USGS or NASA, or LANDSAT satellite images (http://www.landsat.org) that offer global orthorectified Landsat data freely. Moreover, the Global Biodiversity Information Facility (GBIF, http://www.gbif.org/), is an international organisation working to make the world's biodiversity geodata accessible worldwide with the possibility to download livestock species-related data sets.

Finally, the United Nations Environment Programme (UNEP) documented the Global Environment Outlook (http://www.unep.org/geo) — a report presenting the challenges that countries face in safeguarding the environment and for moving towards a more sustainable future - and supplying a data compendium with a list of all key data providers who contributed to the elaboration of the action (http://geocompendium.grid.unep.ch/).

In summary, we do not expect a quantum leap in the short-term. The number of available environmental data bases (new satellites, new environmental monitoring capacities) will gradually increase, their quality will improve (better spatial resolution), and a growing number of geographic areas will be covered. However, most of these global data sets are already freely available and can be used for a comparable description of production environments worldwide.

MOLECULAR DATA

The 2.91 billion base pair (bp) consensus sequence of the euchromatic portion of the human genome was generated by an international consortium of researchers that exploited whole-genome shotgun methods and Sanger biochemistry in a 13-year and $3 billion project (Venter et al., 2001). The recent publication of the complete genome sequence, its annotation, and comparative analysis of the cattle genome by the The Bovine Genome Sequencing and Analysis Consortium (see Elsick et al., 2009) required a six-year effort and a significantly lower budget.

Over the past three years, various innovations of cyclic-array sequencing were introduced into commercial products (e.g. 454 Genome Sequencer –, Roche Applied Science; Solexa – Illumina; SOLiD – Applied Biosystems; Polonator - Dover/Harvard; HeliScope Single Molecule Sequencer - Helicos) and quickly replaced the first generation of Sanger sequencers for genome projects, reducing the cost of DNA sequencing by several orders of magnitude. This promoted the access to these biotechnologies to other groups of users than those restricted to major genome centers or large consortia (Shendure et al., 2004). These second generation sequencing methods operate in the same way: a set of oligonucleotide probes is used to capture the desired sequences from total genomic DNA. These sequences are then amplified in a single PCR reaction using common linkers or adaptors, originally attached either to the probes or to the genomic DNA as primers. Finally, PCR reaction 'enriched' for the desired target sequences is sequenced by synthesis.

In 2004, the US National Institutes of Health (NIH) launched a new challenge to the scientific community: sequence one human genome for US$1000 (Service, 2006). Although this objective has not yet been reached, the latest generation of sequencers (called 'third generation') will make it feasible. In particular, the theoretical potential of single-molecule/nanopore sequencing is undeniable (Tersoff, 2001; Branton et al., 2008). A nanopore-based device enables the detection of nucleobases by electrophoretically driving DNA or RNA molecules in solution through a nano-scale pore without PCR amplification or labeling, thereby providing a unique and inexpensive analytical capability. These 'third generation' instruments offer the prospect of sequencing a diploid mammalian genome for around US$1000 in 24 h (Blow, 2008).

Several alternative low cost sequencing technologies are also under way and even potentially more cost-effective; one example is the Pacific Biosciences technology that claims to sequence a complete diploid genome for less than US$100 (Levene et al., 2003; Eid et al., 2009). This means that in the immediate future, any research project in livestock genetics can take the opportunity of analysing the entire genome of an individual and generate Gigabases of DNA sequence data.

However, in this new era of rapidly evolving technologies and availability of exhaustive data sets, some near-term challenges should be considered to fully exploit the landscape genomics approach: the development of robust protocols for molecular data production, the availability of adequate computational platforms, the development of bioinformatic pipelines for data handling and analysis, and the reformulation of experimental design methods.

Regarding the last topic, large scale sampling of high perfomance standardised livestock breeds and native populations adapted to different endemisms, climates, and management systems should be designed to exploit geographic criteria. Enough biological material should be collected to meet the requirements of the new technologies and appropriate methodologies have to be elaborated or enhanced to carry out whole genome comparative analysis. The overcome of these current challenges will offer a priceless opportunity to detect the genomic regions and genes under adaptive selection or underpinning disease resistance.

COMPUTATIONAL ISSUES

A final and very important issue to enable landscape genomics 'take-off' is the design and validation of a formal methodology and related tools for studying genome-environment relationships. This should include robust sampling strategies across areas of traditional breeding, efficient computer infrastructure to handle whole genome data, as well as eco-climatic parameters, computing resources (computer

grid facilities for instance) to provide enough processing power for a large number of users and easy-to-use software for the analysis and visualisation of the results.

The upcoming whole genome revolution shortened our time to pave the way. With regard to GIS, computational and statistical aspects, the importance must be emphasised of including the recording of geographic coordinates in any new project requiring animal sampling campaigns; it is also necessary to enhance methods for statistical analysis and develop adapted software solutions. Although specific methodological developments (capacity to process ordinal and nominal association models, or to develop spatial statistics for instance) and practical improvements (easy-to-use graphical interface, web-based platform) are still necessary, the calculation process for association models is rather straightforward. Thus, the challenge will mainly consist of improving the efficiency of algorithms, in making software applications usable by both supercomputers and computer grids, and in providing users with a centralised access to analytical tools, as well as to environmental data. These are prerequisites for fully exploiting the opportunity to analyse the complete genome of hundreds of thousands of animals worldwide, and to associate genetic variations with the hundreds of variables that are progressively constituting enhanced environmental data sets. In other words, we must be prepared to handle models potentially composed of millions of single nucleotide polymorphisms (SNPs) and hundreds of eco-climatic parameters corresponding to hundreds of thousands of individuals.

CONCLUSIONS

The landscape genomics approach is promising. Applied to livestock, it should integrate geographical distribution of breeds, their genetic diversity, as well as climatic, ecological, epidemiological and production system information related to the place where animals are reared. It can be used to understand the genetic basis of animal adaptation to the environment, as well as useful information towards optimised breed conservation and management strategies (Long, 2008). It is likely to favour a better management of farm animal genetic resources (FAnGR), as described in the First International Technical Conference on Animal Genetic Resources for Food and Agriculture in Interlaken, 2007 (ILRI, 2007).

Methods, tools and data are now available to detect the footprint of selection driven by environmental parameters. On the basis of existing data, and before the advent of the imminent 'paradigm change in genomics' (see Conference on Next Generation Sequencing: Challenges and Opportunities; http://ngs2009.uab.es/), it is already possible to characterise - to a certain extent - the landscapes to which livestock breeds are best adapted. Together with the integration of global warming models, it is also possible to forecast the consequences of climate changes on breed surviving ability, and to simulate different scenarios for predicting population demographic trajectories and adopting appropriate measures to reduce the risk of extinction. However, forthcoming whole genome data sets will really made us turn to a new dimension of analysis, and therefore lead to new ways to assess genetic diversity. From then on, all conditions will be achieved to enable the real landscape genomics take-off.

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Tandem Inhibin Gene Immunisation to Induce Sheep Twinning

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ABSTRACT

To study the effect of sheep twinning after tandem inhibin gene immunisation, the recombinant plasmid of tandem inhibin were constructed with inhibin α-subunit (1–32) of pig and complement 3d (C3d) of sheep by real-time polymerase chain reactions (RT-PCRs) and used to immunise sheep. The results showed that the recombinant plasmids, pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were constructed successfully. Expression products were secreted after BHK-21 cells were transfected with the recombinant plasmids. After immunisation with 0.4 mg DINH and 0.3 mg DINH-sC3d3, twinning rates were 12.5% and 25.0% respectively, which were significantly higher (P < 0.05) than the control group. However, there was no significant association between twinning rate and immunisation dosage with either antigen. On the basis of these preliminary studies, it is concluded that recombinant plasmids of tandem inhibin gene form a sound theoretical and technical basis for developing an inhibin-based-gene as a vaccine for increasing twinning and reproductive efficiency in sheep. However, further investigations involving more animals are required to determine the most effective dosage and timing of vaccination as well as choice of adjuvant for eliciting an optimal immune response for increasing twinning rates.

Key words: *inhibin, gene immunisation, twinning rate, sheep.*

INTRODUCTION

Inhibin is a type of glycoprotein hormone secreted by testicular sertoli cells and ovarian granulose cells. The protein is structurally a heterodimer composed of two sub-units α and β. Inhibin influences mammalian reproductive performance by regulating secretion of follicle stimulating hormone (FSH) (De Kretser et al., 2000; Medan et al., 2007; Padilla et al., 2007). The development of animal follicles and fertility could be improved by inhibin immunisation. Active immunisation against inhibin increased FSH secretion and ovulation rate in females (Anderson et al., 1998; Medan et al., 2003; Sasaki et al., 2006), and passive immunisation also increased FSH secretions in young adult male Shiba goats (Araki et al., 2000). While the use of active and passive immunisation against inhibin in animal production is restricted by difficulty in preparation and high cost, development of a gene vaccine offers potential to make it practical and effective to improve the reproductive performance of sheep by inhibin immunisation.

Immune technology involving inhibin gene is now becoming a hot research area for improving lambing rates as its efficiency, stability, ease of production and delivery offer potential for circumventing the deficiencies of traditional methods like genetic selection, embryo transfer, superovulation, hormonal induction etc. Different types of inhibin gene vaccines have been constructed and used to immunise mice (Jiang et al., 2002), rats (Mao et al., 2004), sheep (Zhang et al., 2004) and cattle (Cui et al., 2006), with the aim of improving follicular development, ovulation and the number offspring produced, but results to data have been somewhat disappointing. It is therefore necessary to explore novel methods of inhibin production and new strategies involving immunologically-based reproductive technology. To examine the feasibility of developing the inhibin gene as a vaccine for sheep, we constructed a recombinant plasmid of the tandem inhibin gene and investigated its effect on sheep twinning after immunisation.

MATERIALS AND METHODS

Experimental Animals

Sixty adult Gansu Alpin Merino (GAM) ewes were randomly selected from a sheep population of 758 individuals from the Huangcheng sheep breeding enterprise in Gansu Province. These ewes were healthy, being subjected to routine vaccinations and anthelmintic treatments while grazing on wild grassland. The reproduction and twinning rates of the animals averaged 68.3% and 1.3%, respectively.

Gene Sequences and Synthesis

The gene sequence ecoding the α-subunit (1–32) of inhibin in pig was synthesised by AugCT Biotechnology (China, Beijing) and used as the template in the PCR. For this, two pairs of primers containing endonuclease sites were designed according to the gene sequence. The forward inhibin (FINH) gene fragment was obtained from PCR by primers F1 (5'-**AG**GAATTC<u>ATG</u>TCCACCGCC-3', containing a *EcoR* I site) and R1 (5'-**AC**TCTAGA TCTGTGGCAGT C-3', containing a *Xba* I site), while the reverse inhibin (RINH) gene fragment was obtained by primers F2 (5'-**TT**TCTAGATCCA CCGCCCCTCTG-3', containing a *Xba* I site) and R2 (5'-**CG**AAGCTT<u>TTA</u> TCTGTGGCAGTCGGC-3', containing a *Hind* III site). The amplicons of both genes were later cloned into the plasmid vector T-easy via restriction endonucleases and then transformed into host strain JM109. The positive recombinant bacteria were identified and the plasmids extracted. The tandem inhibin gene was obtained through relevant endonuclease digestion and the recombinant plasmid pcDNA-DPPISS-DINH constructed by

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incubating the tandem inhibin gene with pcDNA3.1 and dog pre-proinsulin signal sequence (DPPISS) in a solution of T4 DNA ligase.

The complement 3d (C3d) gene sequence of sheep was amplified by real-time PCR (RT-PCR) from the mRNA in liver tissue and the primers used were designed according to the sequence (GenBank: EF681138) retrieved from GenBank (Yue et al., 2008). The complement 3d gene fragment was inserted into pGEM-T Easy vector and the pGEM-sC3d obtained. The recombinant plasmid pcDNA-DPPISS-DINH-sC3d3 (pcDNA dog preproinsulin signal sequence double inhibin sheep C3d was constructed by linking pGEM-sC3d with pcDNA-DPPISS-DINH.

The recombinant plasmid pcDNA-DPPISS-DINH-sC3d3 was extracted and used to transfect BHK-21 cell line by liposome-mediated transfection. The expression level of fusion proteins DINH and DINH-sC3d3 were determined using Western blot.

Immunisation Schedules

The sixty adult ewes were randomly divided into five groups, each with 12 individuals. Animal treated with pcDNA-DPPISS-DINH were divided into two groups and immunised respectively with doses of 0.2 mg and 0.4 mg. Similarly, sheep transfected with pcDNA-DPPISS-DINH-sC3d3 (two groups) received either 0.3 mg or 0.6 mg.

The control group was injected with 2 mL of saline water. Animals received three gene immunisations at 20-d intervals, the first immunisation being carried out 60 d before mating. Immunisation was by intramuscular injection and artificial insemination was conducted after oestrus.

The results obtained were subjected to SPSS11.5 analysis.

RESULTS

Tandem Inhibin Gene Cloning and Recombinant Plasmid Selection

The forward inhibin (FINH) and reverse inhibin (RINH) gene fragments amplified by PCR were both approximately 115bp (**Figure 1**). Meanwhile, the amplified tandem inhibin gene (DINH) varied between 200bp and 250bp (**Figure 2**), indicating that the two gene fragments amplified were linked together. Thereafter, the tandem inhibin gene (DINH) was subjected to digestion with *EcoR* I and *Hind* III. The two fragments obtained had similar lengths of those obtained by PCR amplification (**Figure 3**).

The recombinant plasmids pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were subjected to digestion with *EcoR* I and *Hind* III, respectively. As shown in **Figures 4** and **5**, the fragments
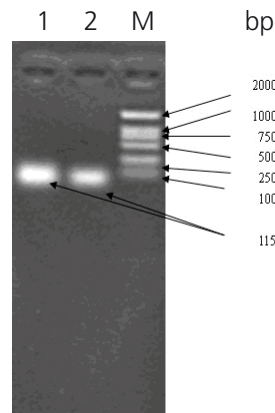
Figure 1. PCR amplification of FINH and RINH. 1 — FINH; 2 — RINH; M — Marker.
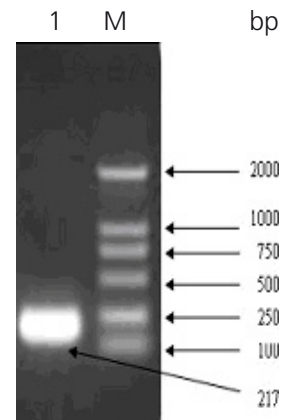

Figure 2. PCR amplification of DINH. 1 — DINH; M — Marker.

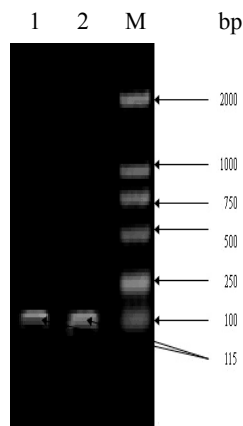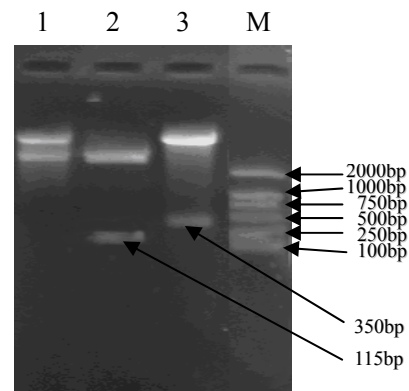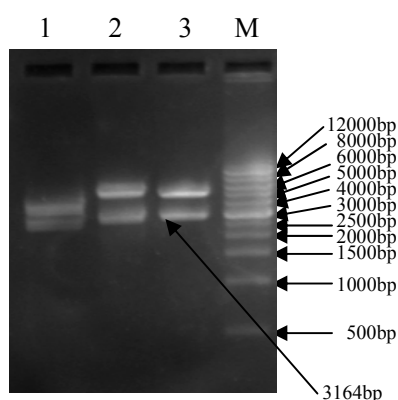**Figure 3. PCR amplification of FINH and RINH. 1 — FINH; 2 — RINH; M — Marker.**

**Figure 4. Identification of recombinant plasmid pcDNA–DPPISS–DINH. M — DNA marker, (2000bp); 1 — pcDNA–DPPISS–INH–C3d3, (*Bam*H I/*Xba* I); 2 — pMD19–INH, (*Bgl* II/*Xba* I); 3 — pcDNA–DPPISS–DINH, (*Hind* III/*BamH* I).**

Figure 5. Identification of recombinant plasmid pcDNA–DPPISS–DINH–sC3d3. M — DNA marker, (500bp ~ 12000bp); 1 — pcDNA–DPPISS–DINH–sC3d3, (*EcoR* I/*Xba* I); 2 — pSG–sC3d3, (*Bgl* II/*Xba* I); 3 — pcDNA–DPPISS–DINH–sC3d3, (*BamH* I/*Xba* I).
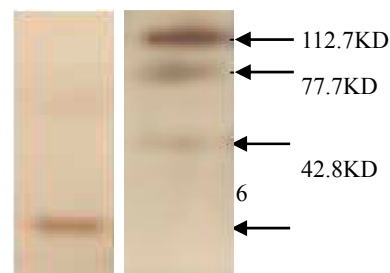
Figure. 6. Western blot analysis of expressed proteins of pcDNA–DPPISS–DINH and pcDNA–DPPISS–DINH–SC3d3 in BHK-21 cells.

obtained were conformed to the design requirements of recombinant plasmids.

Detection of Protein Expression by Recombinant Plasmids

Endonuclease digestion and DNA sequencing confirmed the correctness of recombinant plasmid construction. As indicated in **Figure 6**, the fusion protein DINH of DINH-sC3d3 was expressed in the cell line BHK-21 transfected with the recombinant plasmids pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3. The expressed proteins from the two different constructs showed differences between the two lanes.

Twinning Rate in Immunised Sheep

The results of sheep twinning after tandem inhibin gene immunisation are shown in **Table 1**. Chi-square test indicated that the twinning rates of sheep in the immunised groups were significantly higher (P < 0.05) than those in control group (animals which did not lamb excluded form analysis). Twinning rates were not different between sheep immunised with 0.2 mg and 0.4 mg, but rates in the groups immunised with 0.3 mg and 0.6 mg were significant in statistical difference (P < 0.05).

DISCUSSION

Anderson et al. (1998) immunised Merino ewes with different inhibin alpha subunit peptides conjugated to human serum albumin, and found that immunisation with synthetic inhibin peptides 10–26, 13–26, 7–13, 1–6 resulted in lower inhibin antibody titres and ovulation responses which were associated with increased FSH or ovulation rate, compared with that of longer peptides 1–32, 1–26, 7–16, 8–30. Immunisation with inhibin α-subunit (1–32) has been shown to be better than others (Mayo et al., 1986; Anderson et al., 1998), indicating that the inhibin α (1–32) fragment is an effective antigen when conjugated with a large protein (Mao et al., 2003). In our study, we compared the amino acid sequences of the α-subunit (1–32), mature peptide and precursor protein of inhibins from pigs, cattle, sheep and the mouse. These comparisons indicated higher homologous amino acid sequences in the α-subunit of porcine inhibin than in the α-subunit of bovine, sheep, mouse and rat inhibin; amino acid sequences in α-subunit also showed higher homology with other protein sequences in the pig. In this sense, with the complete gene sequence of the α-subunit of porcine inhibin being used for immunisation, immune-reactions should occur between the antibody produced and other types of protein in the body. However, the amino acid sequence of α-subunit (1–32) not only exhibited immunogenicity of inhibin, but also elicited no immune-reaction with other body proteins. Therefore, its nucleotide sequence is an ideal region for

Table 1. The reproduction rate of sheep in different groups after three immunisations.

Group	Twins	Singles	No lambing	Total	Number of lambs born	Twinning rate
0.2 mg DINH	1	8	3	9	10	11.1[b]
0.4 mg DINH	1	7	4	8	9	12.5[b]
0.3 mg DINH-sC3d3	2	6	4	8	10	25.0[c]
0.6 mg DINH-sC3d3	1	6	5	7	8	14.3[b]
Control	0	8	4	8	8	0[a]

Note: Value in the same column with different superscripts mean significant difference values (P < 0.05); same superscripts mean no statistical difference (P > 0.05).

gene cloning and recombinant plasmid construction. In this work, the recombinant plasmid constructed contained the nucleotide sequence of the N-terminal α-subunit (1–32) of inhibin. Furthermore, we improved the immune efficiency by using two tandem antigen determinants and we expected to improve the reproductive efficiency through gene immunisation by using recombinant plasmids.

As is well known, immune reactions can be promoted by injecting an adjuvant together with the antigen. C3d is a fragment produced by the pyrolysis of C3 during complement activation. As a molecular adjuvant, C3d could decrease the activation threshold of B cells and improve the processing and presentation of antigen. C3d could also promote antibody production and affinity. On the other hand, C3d could transform the immune response from one involving TH1 cytokines to a TH2-type cytokine response, thereby promoting humoral immunity. In this work, the nucleotide sequence of C3d was amplified by RT-PCR, and the primary and advanced structures of cloned gene sequences were predicted using specific computer programs (Yue et al., 2008). By immunising sheep with recombinant plasmids containing the molecular adjuvant C3d, the maximum twinning rate of sheep was as high as 25%, which provides a theoretical and technical basis for developing the inhibin gene as a vaccine for sheep.

Inhibin gene immunisation could counteract the levels of inhibin produced in the body, thereby increasing the level of FSH secreted and in turn promoting multiple ovulation and improving the reproductive performance of mammals (Medan et al., 2007). Currently, research on inhibin gene immunisation involves mainly single-copy gene immunisation (Zhang et al., 2004; Cui et al., 2006), with multiple-copy gene immunisation being rarely reported. However, Cao et al. (2008) constructed the recombinant plasmid of bi-copy inhibin gene (*pcISI*) and successfully used this to immunise rats. After immunisation, the twinning rates of sheep weres 12.5% and 25.0%, respectively, which was significantly higher (P < 0.05) than the control group. We therefore suggest that tandem inhibin gene immunisation can regulate the level of FSH secretion in sheep, promote follicular development and maturation, stimulate multiple ovulation and induce sheep twinning. However, the twinning rates recorded need further improvement, possibly by changing, the dosage of inhibin gene for immunisation and/or the selection of adjuvant.

CONCLUSIONS

In this experiment, the α-subunit (1 to 32) gene in tandem inhibin was cloned. Also, a new type of molecular adjuvant, C3d, was cloned from the liver tissue of sheep. Recombinant plasmids, pcDNA-DPPISS-DINH and pcDNA-DPPISS-DINH-sC3d3 were constructed and expressed successfully. After immunisation, the twinning rates of sheep varied between 12.5% and 25.0% which was significantly higher (P < 0.05) than the control group. The construction of recombinant plasmids of tandem inhibin gene provides the theoretical and technical basis for developing the inhibin gene as a vaccine for sheep.

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Factors Affecting Age of Puberty and the Response of Syrian Female Awassi Sheep to FGA and eCG Treatment

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ABSTRACT

Two experiments were conducted on Syrian female Awassi sheep to characterise certain parameters during various reproductive stages. In experiment 1, 18 ewe lambs were tested at 5 months of age to assess pubertal parameters and affecting factors. The overall average age at puberty was 18.0 months, occurring between May and August (during the normal breeding season). There were no significant differences in time to reach puberty between ewe lambs in terms of the month of birth, type of birth (single or twin) or weaning weight. The average live weight (LWt) and serum progesterone concentration of ewe lambs at puberty were 53.7 kg and 6.32 nmol/L, respectively. A positive and significant correlation (r = 0.72, P < 0.001) was found between progesterone concentration and LWt of lambs. In experiment 2, 16 nulliparous cyclic Awassi ewes, 21 months of age, were treated with intravaginal sponges containing 40 mg of flugestone acetate (FGA) for a period of 14 d during the breeding season. Eight animals (Group P) were then injected intramuscularly at sponge withdrawal with 500 IU of equine **c**horionic gonadotropin (eCG), the remainder (Group C) acting as controls. All females exhibited oestrus and were mated within 3 d of sponge withdrawal. Twinning rates were 37.5% and 12.5% respectively for the animals in Groups P and C (P < 0.05). It is concluded that it is possible to improve the twinning rate of nulliparous Syrian Awassi ewes in their first pregnancy using eCG with no adverse effects on either the ewes or the lambs born.

Key words: *Awassi sheep, puberty, progesterone, intravaginal sponges, equine chorionic gonadotrophin, twinning.*

INTRODUCTION

There is a threshold of LWt necessary for the attainment of puberty in the first breeding season, and when LWt was below that threshold, the first ovulation in Mouflon and Manchega ewe lambs did not occur until the beginning of the next breeding season, despite minimal further growth (Moreno et al., 2000). Galmessa et al. (2003) indicated that Horro ewe lambs tended to breed at similar LWt, but attained puberty at different ages. Nakada et al. (2002) suggested that the development of capacity to secrete LH in response to gonadotrophin-releasing hormone (Gn-RH) before puberty is one of the factors for deciding the time at puberty in heifers.

However, recent research suggests a pivotal role for the hormone leptin (Pittroff, et al., 2008). Leptin has been reported to be required for the normal onset of puberty (Chehab et al., 1997), and to have direct effects through steriodogenesis on the ovary (Ryan et al., 2002). Yu et al. (1997) found that leptin not only stimulates luteinising hormone releasing hormone (LHRH) in the rat but also stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from anterior pituitary cells *in vitro*.

Differences have been reported regarding age at puberty and LWt of ewe lambs in different breeds (Parawan et al., 1987). Also, some researchers found relationships between the onset of puberty and the type of birth (Younis et al., 1978) or weaning weight (Mukasa-Mugerwa et al., 1991), and some have reported no effect of the lambing season on the age of puberty (Lopez-Sebastian et al., 1985), whereas such an effect was reported by others (Papachristoforou et al., 2000).

Synchronisation of oestrus has been recently and widely performed in small ruminants to improve reproductive efficiency and management (Al-Merestani et al., 1999). For this purpose, intravaginal sponges containing synthetic progestagens, namely MAP (medroxyprogesterone acetate) (Kausar et al., 2009) and FGA (flougestone acetate) (Letelier et al., 2009) are used. *Equine chorionic gonadotropin* has been used with the sponge treatment to improve fecundity (Lamrani et al., 2008), and Saloia ewes in Portugal (Silva et al., 2003) and Awassi ewes in Syria (Zarkawi and Soukouti, 2009) treated with eCG had a higher number of follicles over 5 mm in diameter in the ovaries than untreated animals.

The Awassi is a fat-tailed triple purpose and the most important sheep breed in Middle Eastern countries. Its desirable traits, such as the popularity of its meat and milk, high adaptability to different ecosystems, resistance to disease and tolerance to extreme temperature, and endurance of adverse management and feeding conditions (Sleiman and Abi Saab, 1995; Abi Saab and Sleiman, 1995; Salhab et al., 2003) have encouraged breeders in many countries to raise Awassi sheep.

Syrian Awassi sheep (about 23 million, AODA, 2009) are seasonal breeders, mate between June and September (Zarkawi, 1997), and normally lamb once annually. Moreover, they have a relatively poor reproductive performance and a low twinning rate (Thomson and Bahhady, 1988). However, there are no available data on age at puberty in Syrian Awassi ewe lambs using reproductive hormones, such as delineating the age and LWt at which the first elevation in progesterone concentration occurs, followed by a normal oestrous cycle. The availability of such data is essential for studying the reproductive physiology of this breed. Moreover, the effects of intravaginal

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sponges on certain parameters of the young Syrian Awassi ewes in their first reproductive cycle have yet to be determined.

The main objectives of the current study were therefore to determine the LWt and age at puberty in ewe lambs as well as some affecting factors, and to assess the response of nulliparous ewes following insertion of intravaginal sponges and injection of eCG.

MATERIALS AND METHODS

Location and Experimental Animals

The two experiments were carried out at the Division of Animal Production, Der Al-Hajar area, 33 km south-east of Damascus (33°21'N, 36° 28' E; 617 m above sea level). This is a dry area with an annual rainfall of about 100 mm, and in many respects resembles the Syrian steppe region where the majority of sheep are raised.

Animal Housing and Feeding

Animals were kept indoors at night and outside for most of the day. Indoors, the animals were offered diets based on barley and wheat straw supplemented by vitamins (High Vet, Safco Vet Products, Damascus). Outdoors, they had free access to natural grazing. Water and mineral licks (Phosphadin, Al-Shark Vet Products, Damascus) were available *ad libitum*.

Experiment 1

Experimental Animals

Eighteen Syrian Awassi ewe lambs (9 singles and 9 twins), born between December and March were used for a period of 16 months, starting at an age of 5 months and an average LWt of 24.6 ± 4.6 kg. The average birth and weaning weight at 3 months of age of these ewe lambs were 4.7 ± 0.8 and 22.5 ± 5.5 kg., respectively.

Experiment 2

Experimental Animals and Hormonal Treatments

Sixteen cyclic Syrian Awassi ewe lambs (8 singles and 8 twins), aged 21 months and an average LWt of 55.5 ± 6.5 kg., were used for a period of 8 months. Females were randomly allocated in August (during the breeding season) into two equal groups, an experimental (P) and a control (C). Animals in both groups were treated with intravaginal sponges containing 40 mg of FGA (Chronogest®, Intervet International B.V., The Netherlands) for a period of 14 d. However, only females in the P group were injected intramuscularly at sponge withdrawal with 500 IU of eCG (Folligon, Intervet International B.V., The Netherlands).

Oestrus Detection and Mating

Three fertile Awassi rams were introduced daily (08.00 h–14.00 h) into all females in both groups 24 h after sponge withdrawal for oestrus detection and mating (all females were mated within 3 d). Rams were separated from the females until the following day. All females that were in oestrus and mated were recorded.

Blood Sampling and Progesterone Analysis

Blood samples (10 mL) were taken from the jugular vein of all animals twice weekly (at 10.00 h) starting at 5 months of age and continuing for a period of 16 months in the first experiment (n=18) and from the ages of 21 months until 29 months in the second experiment (n=16). Serum was prepared by centrifugation of blood at 3 000 rpm for 20 min., and stored at –20 ºC until assayed using validated progesterone RIA kits (COAT-A-COUNT, DPC, USA). The intra-assay coefficient of variation was 7.2% and the inter-assay coefficient of variation was 7.4%. Progesterone levels equal to or exceeding 3.18 nmol/L were indicative of normal luteal function, while levels under 3.18 nmol/L were indicative of anoestrous, follicular, or the early luteal phases of the oestrous cycle (Zarkawi, 1997).

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using a Statview-IV programme (Abacus Concepts, Berkeley, CA, USA) on an IBM system. In addition, a correlation analysis was used to determine the relationship between blood serum progesterone concentrations and LWt of ewe lambs used in Experiment 1.

RESULTS

Experiment 1

The different parameters related to puberty of the ewe lambs are illustrated in **Table 1**. Based on the first elevation in serum blood progesterone to a concentration exceeding 3.18 nmol/L, as an indicator for active corpora lutea (Zarkawi, 1997), followed by the appearance of regular oestrous cycles as a criterion for the attainment of puberty, it was found that puberty was reached during the second breeding season after birth between May and August at the following rates: May (16.7%), June (27.8%), July (33.3%) and August (22.2%).

The data presented in **Table 2** also indicate that neither the type of birth (singles or twins), nor the month of birth (December, January-March) had a significant effect on the time to attain puberty. Likewise, a high weaning weight of ewe lambs had no significant effect on the age of puberty despite the significant difference (P < 0.05) between the two weights (26.3 kg and 17.8 kg respectively) at three months of age.

Relationship between Serum Concentration of Progesterone and Live Weight

Average serum progesterone concentration and LWt of the lambs during the period from 5 months of age until puberty are shown in **Figure 1**. A positive and significant correlation (r = 0.72, P < 0.001) was found between these parameters during the experimental period.

Experiment 2

Table 3 gives some reproductive parameters for the groups of nulliparous Awassi ewes (P and C), as affected by the eCG intramuscular

Table 1. Live weight, age, and blood serum progesterone concentration (mean ± SD) in 18 Syrian Awassi ewe lambs at 5 months of age and at puberty.

	At 5 months of age	At puberty
Body weight (kg)	24.6 ± 4.6	53.7 ± 7.2
Age at puberty (month)		18.0 ± 1.0
Progesterone concentration (nmol/L)	0.3 ± 0.3	6.3 ± 3.7

Table 2. Effects of litter size, month of birth and weaning weight on age at puberty in Syrian Awassi ewe lambs.

	At 5 months of age	At puberty
Litter size	Single	18.1[a]
	Twin	17.9[a]
Month of birth	Before 1st January	18.4 [a]
	After 1st January	17.6[a]
Weaning weight	High (Mean: 26.3 ± 2.9 kg)	18.3[a]
	Low (Mean: 17.8 ± 3.8 kg)	17.7[a]

Means within a parameter with different superscripts are significantly different (P < 0.05).

Table 3. The effect of intramuscular injection with eCG on some reproductive parameters of the nulliparous Syrian Awassi ewes employed in experiment 2.

Parameter	*Group P (n = 8)	**Group C (n = 8)
Mating weight (kg)	56.0 ± 6.1[a]	55.5 ± 8.0[a]
Mating rate (%)	100[a]	100[a]
Lambing rate (%)	100[a]	100[a]
Weight after lambing (kg)	61.5 ± 6.3[a]	60.8 ± 6.4[a]
Duration of pregnancy (d)	151.4 ± 1.8[a]	151.0 ± 1.5[a]
Twinning rate (%)	37.5[a]	12.5[b]

[a,b] Means, within a row, followed by different small letters are significantly different (P <0.05).

* The experimental group receiving a muscular injection of 500 IU eCG at sponge withdrawal.

** The control group that was not injected with eCG after sponge withdrawal.
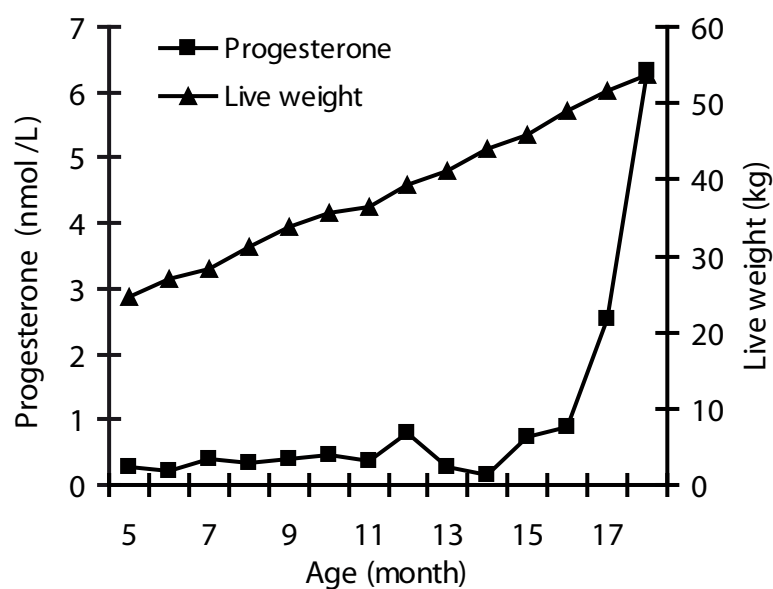
Figure 1. Average blood serum progesterone concentration and mean live weight of Syrian Awassi ewe lambs.

injection. All the females exhibited oestrus and were mated within 3 d after sponge withdrawal (87.5% within 2 d).

Serum progesterone concentration at sponge withdrawal was very low averaging 0.43 ± 0.19 nmol/L. However, this basal concentration increased within 5 d to levels exceeding 3.18 nmol/L, remained high throughout pregnancy to term and decreased sharply to concentrations below 2.0 nmol/L just after lambing.

Twinning rate was 37.5% in animals that received eCG (Group P) as compared with 12.5% for those in the control group C; the difference was significant ($P < 0.05$).

There were no problems during delivery and the lambs born together with their mothers were healthy; mortality from birth to weaning at 3 months of age was zero in both groups.

DISCUSSION

This study provides additional information on the age at puberty in local Awassi female sheep as well as other related information and adds to previous observations (Zarkawi et al., 1999; Zarkawi, 2000; Zarkawi, 2004).

Blood progesterone concentrations have been widely used by researchers in many countries as a valuable indicator to monitor the age at puberty in some animal species, such as Shiba goats in Japan (Sakurai et al., 2004), Braford and Brahman x Angus heifers in the USA (Cooke and Arthington, 2009), Murrah buffaloes in India (Haldar and Prakash, 2005), and in some sheep breeds such as Charollais x Awassi, Romanov x Awassi in Jordan (Kridli et al., 2006) and Karagouniko in Greece (Valas et al., 2006).

Like in many other small ruminants, to attain puberty in Awassi ewe lambs, targets in both age and LWt have to be achieved, since both are involved in activating the Gn-RH pulse generator in the brain and trigger puberty (Adam and Robinson, 1994). Ewe lambs in the current study did not reach puberty during the first breeding season (at 6 - 9 months of age). This can be explained by the animals' failure to reach a threshold of LWt and/or age necessary for the attainment of puberty in the first breeding season. The onset of puberty is associated with an increased frequency of luteinising hormone (LH) pulses, stimulating follicular development, a sustained increase in oestradiol secretion, a preovulatory LH surge, and ovulation (Foster et al., 1985). In ewe lambs below the LWt threshold, initiation of frequent LH pulse secretion is inhibited (Rhind, 1992).

Based on the first rise in serum blood progesterone concentration >3.18 nmol/L, followed by a regular oestrous cycle, the average LWt and age at puberty in the current study were 53.7 kg and 18.0 months, respectively. Using a similar criterion, Abella et al. (2005) reported that the age and body weight at puberty was similar in three genotypes of ewes (Fec^{B}Fec$^+$, Fec$^+$Fec$^+$ Booroola x Merinos d'Arles and Merinos d'Arles) (332.5, 334.8, 330.8 d and 34.1, 34.1, 34.9 kg, respectively).

Breed-related differences in both the age and LWt of ewe lambs at puberty have been reported. Parawan et al. (1987) reported an average of 13.2 months and 19.5 kg at puberty in Philippine ewe lambs, whereas the corresponding figures for Iranian Mehraban ewe lambs were about 8 months and 44 kg (Bathaei and Leroy, 1997). Kridli et al. (2006) reported that crossing Awassi ewes with either Charollais or Romanov sires in Jordan improved the reproductive characteristics of the F_1 crossbred by advancing age at puberty.

The present data indicate that ewe lambs born between December and March reached puberty in the same breeding season indicating that there was no effect of lambing month on the attainment of puberty. Spanish Mouflon lambs born in March/April and that reached a minimum threshold body weight (23.8 kg) in their first breeding season reached puberty at 8 months of age whereas in

those with slower growth rates, the prepubertal period was extended throughout the first breeding and non-breeding seasons, with puberty being reached during the breeding season of the following year at 19 months of age and 27 kg body weight (Santiago-Moreno et al., 2001).

In our study, all females treated with FGA plus eCG showed oestrus behaviour and were mated within 3 d after withdrawal of the sponges, became pregnant to term and lambed normally with no adverse effects on either themselves or their lambs. This indicates that nulliparous Syrian Awassi ewes could respond to the above treatments at an early age (21 months) with no effect on mating rate, duration of pregnancy, health of lambed ewes and lambs born or on the weight of the lambs. Similar results were reported by Zarkawi (2001) on adult Syrian Awassi ewes treated with MAP + eCG. Hamra et al. (1988) treated Iraqi Awassi ewe lambs aged 8–10 months with intravaginal sponges, and found that 77% of the treated lambs showed oestrus behaviour and were mated, but none of them became pregnant, indicating that they had either not attained the proper LWt and/or were not old enough; thus, the ovarian follicles had not reached the preovulatory stage.

Syrian Awassi ewe lambs responded in their first mating to 500 IU of eCG injection, twinning rates increasing from 12.5% in untreated ewe lambs to 37.5% in eCG-injected ones. Most probably this would have a positive impact on farmers' incomes. The fact that all ewe lambs started cycling after FGA + eCG treatment without problems is a further advantage of using such treatment. An increase in twinning rate from 20% in sponge-treated adult Syrian Awassi ewes without eCG intramuscular injection to 50% in eCG-injected ewes was reported by Zarkawi (2001) and using a similar procedure (sponges + eCG), lambing rates increased from 153% in untreated to 206% in treated Suffolk ewes (Tetuska et al., 1988) and from 100% to 134% in Karaman, Tuj and Turkish Awassi (Atsan et al., 2007).

In the current study, there was no significant difference in the duration of pregnancy between treated (151.4 d) and untreated (151.0 d) young ewes. A similar duration of pregnancy (152.0 d) was reported by Zarkawi (1997) in untreated adult Syrian Awassi ewes during the breeding season, confirming that the hormonal treatment had no effect on the duration of pregnancy.

CONCLUSIONS

Syrian Awassi ewe lambs attained puberty stage during the second breeding season after birth at an age of about 18 months and a LWt of around 54 kg. The month of lambing, birth weight, type of birth and weaning weight had no effect on the attainment of puberty.

Nulliparous Syrian Awassi ewes responded well to hormonal treatments (FGA + eCG) in terms of oestrus synchronisation and mating, with no effect on the duration of pregnancy, birth or weaning weight. Injection of eCG could be safely employed to improve twinning rate.

ACKNOWLEDGEMENTS

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Reproductive Performance following Artificial Insemination in Sanga and Crossbred (Friesian × Sanga) Cows in the Accra Plains of Ghana

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ABSTRACT

The performance records of Sanga and Friesian-Sanga crossbred cows kept at the artificial insemination (AI) Centre of the Amrahia dairy farm in the Accra plains of Ghana between the period January 1998 to December 2007 were evaluated. The intervals from calving to first AI, calving to conception, and calving interval were prolonged especially in the Sanga cows inseminated with Friesian semen, averaging 158.8±8.9, 177.5±9.5 and 517.9±13.5 d respectively. In Friesian-Sanga cows bred with Friesian semen these intervals were respectively 115.7±19.2, 138.7±16.3 and 510.3±41.0 d. These parameters were not affected (P > 0.05) by season of calving preceding AI and season of insemination. The conception rates at first AI service and for all inseminations were low in the Sanga (42.6% and 46.0% respectively) and in crossbred cows (53.5% and 53.4% respectively). They were not affected (P > 0.05) by the season of insemination. Improving the nutritional status of the cows through strategic supplementation coupled with effective heat detection techniques, appropriate timing of AI, as well as efficient methods of storage, transport and handling of semen should improve the reproductive performance of cows.

Key words: *reproductive performance, artificial insemination, Sanga, Friesian × Sanga, conception, calving intervals.*

INTRODUCTION

The Ministry of Food and Agriculture in Ghana began a five-year National Livestock Services Project in 1994 with the objective of increasing meat and milk production through breed improvement using AI to meet the protein needs of the population as well as reduce the country's increasing dependence on livestock and livestock products. The introduction of AI for breed improvement has, however, met some difficulties in Ghana. These include lack of appropriately designed breeding programmes and technical shortcomings including poor management practices, inadequate nutrition and occurrence of reproductive disorders.

A major factor affecting the success of AI is the conception rate which in turn is influenced by several factors and their interactions including those related to the cow, management of animals, AI services, semen quality, bull fertility (Nordin et al., 2007) and high environmental temperatures or heat stress (Chebel et al., 2004). The main objective of this study was to evaluate the reproductive performance of Sanga and Friesian-Sanga (crossbred) cows kept at an AI Centre. This would enable the development of measures to improve the efficiency of the AI service provided to cattle farmers.

MATERIALS AND METHODS

Location of Experiment

The study was based on AI carried out between the period 1998–2007 on Sanga and Friesian × Sanga (crossbred) cows kept at the AI Center of the Animal Production Department's Amrahia dairy farm located at latitude 05° 46′ N and longitude 00° 08′W in the Accra plains of Ghana. Total rainfall for the study period was 900.9 mm with an average daily temperature of 29°C. Rainfall was bimodal with peaks in June and October, April to July being the major rainy season, and September to November the minor rainy season. The driest months were January–March, August and December.

Management of Animals

The Sanga cows were grazed from 08.00 h–15.00 h on natural pastures comprising *Panicum maximum*, S*tylosanthes haemata, Sporobolus pyramidalis* and *Vertiveria fulvibarbis* which constitute the dominant grass species in the grazing area. They had access to water from a dam twice daily in addition to water provided in the animal house *ad lib.* The crossbreds were zero grazed. They were provided with *Panicum maximum,* sorghum and spent malt, in addition to a concentrate mixture based on maize, wheat bran, palm kernel cake with or without soyabean meal. Salt lick was always provided. The crossbreds had access to water in the animal house *ad lib.* Oestrus (heat) was observed for the two groups of cows twice daily at 06:00 h and 18:00 h. A cow standing to be mounted (standing heat) was used as the main criterion for the cow to be on heat and therefore ready for insemination. Cows observed to be on heat in the morning were inseminated in the evening of that day, while those which demonstrated signs of heat in the evening were inseminated the following morning. Friesian semen was used for insemination.

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The semen was imported from South Africa, and kept frozen under liquid nitrogen. Four sires were used in the AI programme.

Data Collection

Artificial insemination records from 126 Sanga and 35 Friesian × Sanga cows were used. These covered a 10-y period (January 1998 to December 2007). Parameters studied included interval from calving to first AI, interval from calving to conception, calving interval and conception rate. The effect of season of calving preceding AI and season of AI on calving to first AI, calving to conception, calving interval and conception rate were evaluated.

Conception rate at first service was estimated from the following equation:

$$\text{Conception rate at first service} = \frac{\text{No. of conceptions at first service}}{\text{Number of first services}} \times 100$$

$$\text{Conception rate} = \frac{\text{Number of conceptions}}{\text{Number of services}} \times 100$$

Statistical Analyses

The general linear models (GLM) procedure of the Statistical Analysis Systems Institute (SAS, 1999) was used in investigating d from calving to first AI, d from calving to conception, calving interval and the effect season of calving preceding AI and season of insemination on these parameters. The following model was applied:

$$Y_{ijk} = \mu + S_i + C_j + e_{ijk}$$

where Y_{ijk} = d from calving to first artificial insemination, d from calving to conception, calving interval.
μ — overall mean
S_i — effect of ith season of calving preceding AI
C_j — effect of the jth season of insemination
e_{ij} — a random error associated with each observation

Differences between means were tested by PDIFF/SAS.

The effect of season of insemination on conception rate was assessed using the Chi-square test.

RESULTS

Calving Intervals

The overall mean intervals from calving to first AI, calving to conception, and calving interval in the Sanga cows were 158.8±8.9, 177.5±9.5 and 517.9±13.8 d respectively. These parameters were not affected (P > 0.05) by season of calving preceding AI and season of insemination.

The overall mean interval from calving to first AI averaged 115.7±19.6 d in the Friesian × Sanga crossbred cows, while the mean interval from calving to conception was 138.7±16.3 d and the calving interval averaged 510.3±41.0 d. Neither season of calving preceding AI nor season of insemination influenced (P > 0.05) these variables.

Conception Rate (CR)

The CR at first service for the Sanga cows was 42.6%, and for all services it was 46.0%. The number of services per conception averaged 2.3. Season of insemination did not affect (P > 0.05) conception rate and number of services per conception.

The CR at first service for the crossbred cows was 54.5% and for all services it was 53.5%. The mean number of services per concep-

tion was 1.9. The season in which cows were inseminated did not affect (P > 0.05) conception rate and number of services per conception in crossbred cows.

DISCUSSION

The average calving to first service intervals of 158.8±8.9 d and 115.7±19.2 d obtained in this study for the Sanga and crossbreds respectively were long compared with periods considered to be economically desirable. This delay of first service after calving, particularly in the Sanga cows, may be due to prolonged postpartum anoestrus (interval from calving to the resumption of ovarian cyclicity), most likely a result of inadequate nutrition and suckling management (Jolly et al., 1995; Diskin et al., 2003).

The Sanga cows were grazed mainly on natural pastures and were not supplemented with either crop residues, agro-industrial by-products or energy or protein concentrates. During the dry season, the limited pasture available on the Accra plains is of poor quality; protein levels are low and the grasses become fibrous and highly lignified affecting their digestibility. In addition, there was lack of restriction on suckling by calves, cows being allowed to suckle their young until they were weaned naturally between six and nine months of age (Obese et al., 1999 and 2009). The low nutritional status of animals coupled with the prolonged suckling stimulus could delay normal resumption of ovarian cycles by interfering with the synthesis and secretion of hormones especially luteinising hormone and insulin-like growth factor-I which are important in ovarian follicular development and function in cattle (Williams et al., 1996; Diskin et al., 2003; Thatcher et al., 2006). Poor heat detection and silent heat could be additional factors accounting for the prolonged intervals from calving to first service in both the Sanga and crossbred cows.

The interval from calving to first AI was more prolonged in the Sanga cows and this may account for their extended calving to conception intervals (177.5±9.5 d) compared with the crossbred cows (138.7±16.3 d). Furthermore, the prolonged interval from calving to first AI obtained for the Sanga and crossbreds may have contributed to the extended calving to conception and calving intervals. Eduvie and Oyedipe (1991) reported that the main determinant of long calving intervals is a prolonged postpartum anoestrous interval.

The calving to conception and calving intervals obtained for Sanga cows inseminated with Friesian semen in this study were higher than the 155.2±4.5 d and 444.3±16.5 d respectively reported for the Sanga breed on smallholder peri-urban dairy farms on the Accra plains of Ghana (coastal savanna zone) (Obese et al., 1999). They were also higher than the values of 149.7±5.8 d and 43±6.7 d reported for the same breed on smallholder farms in the humid forest zone in Ghana (Osei et al., 1993).

The extended overall mean estimates for calving to first service, calving to conception and calving intervals obtained for the Sanga and crossbred animals studied here are unfavourable for profitable livestock production. Better management practices including improving the nutrition of cows by strategic feed supplementation especially during the dry season, as well as early weaning or restricted suckling of calves should shorten the postpartum anestrous period and subsequently reduce calving to conception and calving intervals in these herds. Treatments to synchronise oestrus can provoke an increase in plasma LH concentrations and hasten the onset of ovulation and ovarian cycles and thus improve the efficiency of AI programmes. Results from this study indicated that generally the reproductive performance of animals at the Amharia farm was lower than in cows owned by smallholder farmers in the Accra plains. This may be due to the fact that, whilst the private farmer seeks to maximise profit and therefore strive to provide adequate resources for farming, state-

owned farms tend to suffer from bottlenecks including the lack and timely release of funds and resources for farm operations. This tends to delay the implementation of the kinds of interventions suggested above for improved animal productivity.

The overall CR to first service was poor especially in the Sanga cows. The major reason for this low CR may be poor heat detection, inappropriate timing of AI, poor insemination technique or poor semen quality. The timing of insemination in relation to first detection of heat is critical for achieving high conception rates (Peters and Ball, 1995; Tjiptosumirat et al., 2007) as are factors relating to the transport, storage, handling and thawing of semen in the field (Peters and Ball, 1995). Putting in place very effective heat detection mechanisms could reduce undetected oestrus, while more appropriate timing of AI coupled with better transport, storage, handling and thawing of semen should improve the conception rate of cows.

CONCLUSIONS

The intervals from calving to first service, calving to conception and calving intervals were prolonged in Sanga and Friesian × Sanga crossbred cows kept on a government farm in Accra Plains of Ghana. Possible factors involved include prolonged postpartum anoestrus, a consequence of poor nutrition and suckling management especially in the Sanga cows. Conception rates were poor, probably due to one or a combination of management factors including poor heat detection, inappropriate timing of AI, poor insemination technique and low semen quality.

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Evaluation of Semen Quality of Three Boar Genetic Lines Reared in Intensive Units in Romania

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ABSTRACT

This paper reports the results of studies to establish the reproductive performance of breeder lines belonging to one of the most important providers of genetic material in Romania (the Pig Improvement Company, P.I.C.), within a top swine husbandry unit in Romania. Parameters measured in three PIC boars lines (PIC 1075, PIC 402, PIC 408) were sperm volume, spermatozoal concentration in sperm, total numbers of spermatozoa, number of doses produced etc. Ejaculate volumes ranged between 224 mL and 235 mL at between eight and 12 months of age, between 310 mL and 366 mL at 13–24 months old, between 330 mL and 348 mL between 25–36 months old and between 304 mL and 404 mL when 37–42 months of age. There were significant differences between both boar genetic lines and age periods. Comparing levels of spermatozoa in the semen of boar lines, PIC 1075 had an average of 372×10^6 spermatozoa/mL and PIC 402 had 311.5×10^6 spermatozoa/mL, a difference of 16%, or compared to PIC 408 (302.3×10^6 spermatozoa/mL) a difference of around 19%. The highest number of doses (21) was produced by the PIC 402 and PIC 1075 lines, but differences between groups were not significant. It is concluded that due to the high sperm concentration per ejaculate throughout the exploitation period, the use of the PIC boars studied could be improved by decreasing the interval between sampling.

Key words: *boar, genetic lines, sperm, ejaculate volume, motility.*

INTRODUCTION

Alignment with European standards requires pigs breeders in Romania to adopt new strategies for increasing the national swine herd and improving its genetic potential for increasing meat production. Especially important is putting in place systems to maximize exploitation of the genetic potential of hybrid stock in Romania.

The aim of genetic selection is to improve performance and ultimately profitability by incorporating the beneficial traits from a breed type while eliminating undesirable traits. Terminal sire lines have been shown to affect the reproductive traits of the sows with which they are mated. Young boars are used for reproductive activity from the age of 10–12 months until they are 36–42 months old. In some cases, however, they begin their reproductive activity at the age of 8–9 months (Bogdan et al., 1999; Păsărin, 1997; Stoica, 2003) with good reproductive performances.

The principal objective of this study was to examine the quality of semen produced by different boar types available in Romania at different ages.

MATERIALS AND METHODS

The study was carried on 15 boars, each line (PIC 1075, 402 and 408) being represented by five boars. The semen obtained from the 1075 boars is used to artificially inseminate PIC 1050 sows from the hybridisation farm of the unit, whereas semen from the boars of PIC 408 and PIC 402 lines, is used to artificially inseminate Camborough sows, with the resulting piglets earmarked exclusively for slaughter.

Accommodation and other conditions were similar for all boars and the age differences between them were minimal. Semen quality of the boars was assessed from the onset of reproductive activity until culling. The three first weekly series of ejaculations obtained when the animals were eight months old were not evaluated. Intensity of use was semen collection at 9 a.m followed by 5 d resting. A metallic dummy (1 m long and adjustable in height) was used for collecting semen using the gloved-hand technique.

After collection, each ejaculation was submitted to quantitative and qualitative evaluation in the company laboratory. Measurements included: ejaculate volume (ml), motility (percent), sperm concentration ($\times 10^6$/mL), sperm/ejaculate ($\times 10^9$), number of doses/ejaculate.

Semen concentration was determined using a Spermaque photo densitometer, motility was assessed by microscopy and based on the proportion of spermatozoa which moved straight forward. The semen extender used for preparing doses was the XCLL.

Age groups used for data analysis were between 8–12 months, 25–36 months and 37–42 months, when the boars were culled.

RESULTS AND DISCUSSION

Knowledge of the yield dynamics and quality of boar sperm allows specialists to optimise its use for maximising the production potential of the flock. The results obtained for the boar genetic lines investigated here are shown in **Table 1**.

Ejaculate volume of the three bloodlines of boars fell within the limits described by others (Feredean, 1974; Păsărin, 1997; Bogdan et al., 1999). It reached values that ranged between 224 mL and 235 mL when the animals were 8–12 months old, between 310 mL and 366 mL at 13–24 months, between 330 mL and 399 mL at 25–36

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Table 1. Semen characteristics of three PIC boar genetic lines reared in intensive units in Romania.

Variable[1]	Line[2]	Age (months)				Mean
		8–12	13–24	25–36	37–42	
Volume (mL)	1075	232±8[a]	310±7 [b]	330±8[c]	304±7[b]	294
	408	224±7[a]	358±9[b]	383±7[c]	404±12[d]	342
	402	235±8[a]	366±8[b]	399±9[c]	406±14[c]	352
Motility (%)	1075	75.4[a]	78.6[a]	77.3[a]	76.4[a]	76.9
	408	78.7[a]	79.9[a]	79.4[a]	77.8[a]	78.9
	402	78.8[a]	80.4[a]	80.3[a]	79.0[a]	79.4
Concentration (millions/mL)	1075	351±8[a]	372±9[b]	420±8[a]	346±9[c]	372
	408	286±14[a]	298±8[acd]	320±9[bc]	305±13[d]	302
	402	292±13[a]	307±7[b]	331±7[c]	316±13[b]	312
Sperm per ejaculate ($\times 10^9$)	1075	94±12	119±14	143±11	107±12	116
	408	61±12	110±12	123±11	124±13	105
	402	70±10	116±12	129±12	128±11	111
N° doses/ejaculate	1075	17±6	21±9	24±8	20±7	21
	408	12±5	21±6	23±6	23±6	20
	402	16±6	20±8	25±7	23±7	21

[1] Semen was collected at 5-d intervals; [2] Five boars per genetic line.

[a,b,c,d] Means with different superscripts are statistically different ($P < 0.05$).

months old and between 304 mL and 406 mL at 37–42 months old. Significant differences were apparent between both boars and age periods. For example, an upward trend with age of animal was observed in all three lines, and the maximum volume of semen was produced between 25–36 months by PIC 1075 boars and after three years by those from the PIC 402 and PIC 408 line. The finding of lowest values in all three lines at the beginning of reproduction activity is in accordance with the values presented in the literature, and the fact that the function of male genital organs and age of breeding stock are closely inter-related (Feredean, 1974; Bogdan et al., 1999; Nacu, 2005). Considering the whole reproductive life, best results were obtained in the PIC 402 bloodline. The difference between PIC 402 (352 mL) and PIC 1075 (294 mL) was 16.5%, but relative to PIC 408 the difference was only 2.8%.

Motility is also an important quality characetristic of semen, but significant differences were not recorded between the three PIC boar lines or with age, although highest motility was recorded at 13–24 months of age. Expressed in relative values, the differences between the average level observed in PIC 402 line (79.4%) and those found in the other lines, were very minor (3% and 0.6% respectively for the PIC 1075 and PIC 408 lines). The values for spermatozoal motility registered in the boars studied here are similar to those recorded earlier from different synthetic lines and pure breeds (Thibault and Levasseur, 1991; Watson and Behan, 2002).

The concentration of spermatozoa in semen is the main parameter used to dilute semen for insemination. Spermatozoal concentrations varied with age in the three PIC lines, with lowest concentrations being recorded at 8–12 months and highest between the ages of 25 and 36 months. This was probably a reflection of the intensification of spermatogenesis from the onset of sexual maturity associated with appropriate feeding and husbandry conditions, while the decrease noted at 37–42 months was the result of the slowing down of spermatogenesis function after the age of three years. Comparing the values recorded in each boar line, the differences between

PIC 1075 (372×10^6 spermatozoa/mL) and PIC 402 (312×10^6 spermatozoa/mL) and PIC 408 (302×10^6 spermatozoa/mL), were 16% and 19% respectively.

Although the conditions of husbandry and semen collection were similar during the whole exploitation period, the concentration of spermatozoa in ejaculations fluctuated greatly from one collection to another, with differences occurring between periods and lines being significant or highly significant. For example, the maximum number of spermatozoa from an ejaculate (143×10^9) was registered between 25–36 months of age, and the minimum (94×10^9) from the age of 8 months - 1 year. In the PIC 408 boars, the average ejaculate contained 105×10^9 spermatozoa, with the maximum (124×10^9) being registered during the 36–42 months age period, and the minimum (66.1×10^9) from the age of 8–12 months.

Earlier publications quote lower concentrations i.e. between 211 and 315 million spermatozoa/mL (Popovici et al., 1980; Bogdan et al., 1999), than reported more recently e.g. 480–690 million spermatozoa/mL (Kunk et al, 2001; Stoica, 2003; Sgura et al., 2008). However, in the PIC 1075 boars the average number of spermatozoa/ejaculate was 116×10^9 i.e. much higher than any of the above. Such differences may have arisen because the harvesting frequency in the present study was one collection followed by 5 d of rest.

The number of doses issued from each ejaculate was calculated on the basis of a minimum 75% spermatozoal motility and to provide four billion spermatozoa/insemination dose. For the genetic lines studied here, the maximum number of doses/ejaculate was achieved in all three PIC bloodlines during the period when the boars were 25–36 months of age, while the lowest number of doses was obtained during the onset of reproductive activity (i.e. at 8–12 months of age). The highest number of doses (21) was produced by the PIC 402 and 1075 lines, but differences between lines were not significant.

CONCLUSIONS

Analysis of quantitative and qualitative variables relating to the semen of three lines of PIC breeding boars showed that sperm production both quantitatively and qualitatively was in accordance data presented in the literature. However, values for the main indices fluctuated with both age and the line of boar, indicating that opportunities exist for improving boar management to improve productivity.

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Progesterone Levels in the Ovarian, Uterine, and Systemic Venous Blood in Alpacas with Embryo Mortality

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ABSTRACT

Embryo mortality was studied in a group of 20 pregnant alpacas. The reproductive organs of the females were monitored by ultrasound examination to determine signs of sustained pregnancy or embryo mortality. Blood samples were collected from the jugular vein twice weekly from mating to determine progesterone levels through gestation or until the occurrence of embryo mortality. Ovarian hysterectomy was conducted in four animals at day nine post-mating, in three animals at the time of embryo mortality detection and in two others at day 73 of gestation. Blood samples from the ovarian and uterine veins were collected during the surgery and prior to hysterectomy for progesterone determination. The remnant of embryo membranes and the uterus and ovarian structures were macroscopically examined after surgery. The three cases of embryo mortality occurred at days 19, 40 and 69 of gestation. Progesterone levels were high during the process of embryo mortality.

Key words: *alpaca, embryo mortality, progesterone, ultrasound, ovarian veins, gestation.*

INTRODUCTION

The harsh environmental conditions of the highlands of Peru and Bolivia limit agricultural activities, including livestock production. The South American camelids, especially the domestic species of alpaca and llama are suitable options for large commercial farmers, community farmers, and peasants. Fibre and meat are the main animal products, but manure is used for heating and cooking, and llamas are used to carry products from and to markets. Conception rate is adequate in all camelid species during the 3–4 month breeding season, but unfortunately embryo mortality can be as high as 50% (Fernandez Baca, 1970).

Several studies have attempted to identify the main factors involved in embryo mortality but most have focussed on independent factors without much success. Studies on possible relationships between age, pathogenic agents, and genital tract alterations in relation to embryo mortality are scarce and unreliable. Embryo losses are affecting genetic programmes and breeding systems as overall productive performance cannot meet the expected goals.

Embryo mortality in the alpaca outside the Andean region is much lower, but still, New Zealand reported 24% embryo mortality between 21 d and 30 d of gestation some years ago (Ridland et al., 1993). It may be that specific factors related to the high altitude, nutritional deficiencies, and local pathogens are affecting these indigenous animals. Nutritional restrictions decrease growth rate and follicular size affecting the ovulation of the single dominant follicle (Mackey 1999), and this would be related to leptin release as this indirectly regulates gonadotropin-releasing hormone neuronal function (Quennell et al., 2009). Also, it has been reported that cows fed with diets rich in energy produced smaller but better quality follicles than cows fed with low energy diets (Boland et al., 2001).

Several pathogens have been reported to cause embryo mortality, among them being *Toxoplasma gondii* (Gorman et al., 1999), *Neospora* (Serrano-Martínez et al., 2007), bovine viral diarrhoea virus (Carman et al., 2005), and bacteria involved in uterine infections as consequence of retained placenta, dystocia, and vaginal or uterine prolapse (Tibary, 2006).

Oestradiol has been associated with maternal recognition in alpacas (Chipayo, 2003). Females that received estradiol on d 9 and 11 after ovulation had corpora lutea with extended lifespan and showed increased serum progesterone levels (Powell, 2007). It is also known that progesterone increase in the late luteal phase is associated with smaller and less viable embryo in ewes (Mann et al., 1996). According to Boland et al. (2001), there is no relation between peripheral serum levels and ovary-uterus circulation levels of progesterone; meaning that embryo survival in camelids would be more related to progesterone levels in the ovarian and uterine veins than variations of progesterone levels in peripheral blood.

The objective of the present study was to relate progesterone levels in the uterine-ovary circulation and in peripheral circulation in pregnant alpacas that have maintained the gestation or lost the embryo.

MATERIALS AND METHODS

Twenty non-pregnant multiparous female alpacas without calf at foot were selected for this study. The animals were kept in corrals at the Veterinary Faculty of Cayetano Heredia University in Lima, at sea level. They were fed with alfalfa hay and sustained a body condition score of 3 (Australian Alpaca Association, 2001). Two adult and fertile males were used for natural mating.

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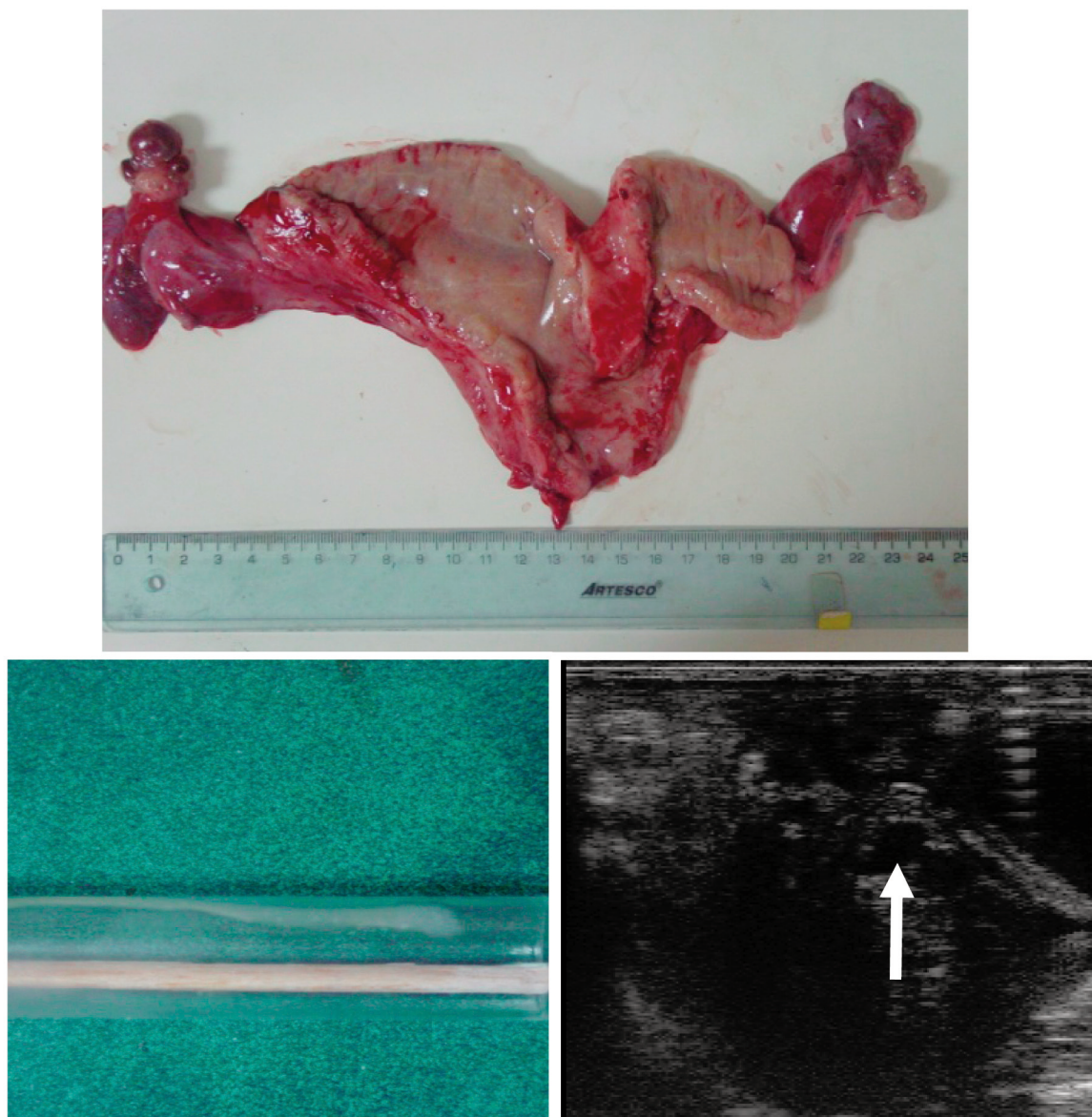
Figure 1. First case of embryo mortality. The upper picture shows the uterus and ovaries. The picture on the left shows the chorionic membranes, and on the right the echographic image of the uterus is displayed. The presence of anechoic content in the uterus five d prior to the embryo loss is shown by the arrow.

Table 1. Serum progesterone levels in alpacas of various reproductive statuses.

Reproductive status	Days post breeding	Progesterone (ng/mL)		
		Jugular vein	Ovarian vein	Uterine vein
Dioestrus	9	2.2±0.4	67.5±12.1	5.9±0.5
Normal gestation	73	4	68.3±8.9	4.7
Embryo mortality (Case 1)	19	3.4	65.3	4.2
Embryo mortality (Case 2)	40	0.4	8.7	1.4
Embryo mortality (Case 3)	69	0.5	4.8	1.1

Copulation was allowed when females showed sexual receptivity and had an 8 mm follicle in any of the ovaries based on ultrasound examination (Bravo, 1991; Vaughan, 2004). Female sexual behaviour was evaluated 13 d after mating and receptive females were mated again.

Pregnancy was monitored by ultrasound examination every other d in 16 animals starting on d 15 until signs of embryo mortality occurred or pregnancy continued until d 90. Embryo mortality was considered to have occurred when embryo cardiac beat decreased, embryo motility was lost, or suspended particles appeared in foetal fluids (Ginther, 1985; Adams, 1989; Parraguez, 1997).

Ovarian hysterectomy was performed in animals as soon as they showed signs of embryo mortality, in four animals chosen at random on d 9 post-mating and in two animals at d 73 of gestation. The anaesthetic protocol included ketamin 10%, tramadol 0.1%, xilacine 20% and atropine 0.03% (Hinostroza, 2010). Exposure and resection of uterus and ovaries were by laparoscopy with a 10 cm skin incision cranial to the mammary gland in the ventral midline (Mendoza et al., 2007). Uterus and ovaries were evaluated macroscopically.

Blood samples for progesterone determination (5 mL) were taken from the jugular vein twice weekly from the d of mating until surgery or 90 d after mating. Also, blood samples were collected prior to hysterectomy from both uterine and ovarian veins (left and right). Blood samples were centrifuged at 3 000 rpm for 10 min and then, serum was harvested and kept at –20 °C until analysis.

Progesterone concentration in serum samples were measured by radioimmunoassay (Coat-A-Count Progesterone In-vitro Diagnostic Test Kit). The standards were 0, 0.1, 0.5, 2, 10, 20, 40 ng/mL, and the coefficient of variation was 5.5% and 1.5% for the high and the low control sample respectively. Progesterone values from the uterus and ovarian vein of hysterectomised animals on d 9 were used as dioestrus values and those at the end of the trial in pregnant animals as normal gestation values.

RESULTS

Three cases of embryo mortality were found during the study. In the first case, embryo mortality occurred on d 19 of gestation (**Figure 1**). Macroscopically, chorionic membranes were observed in the left
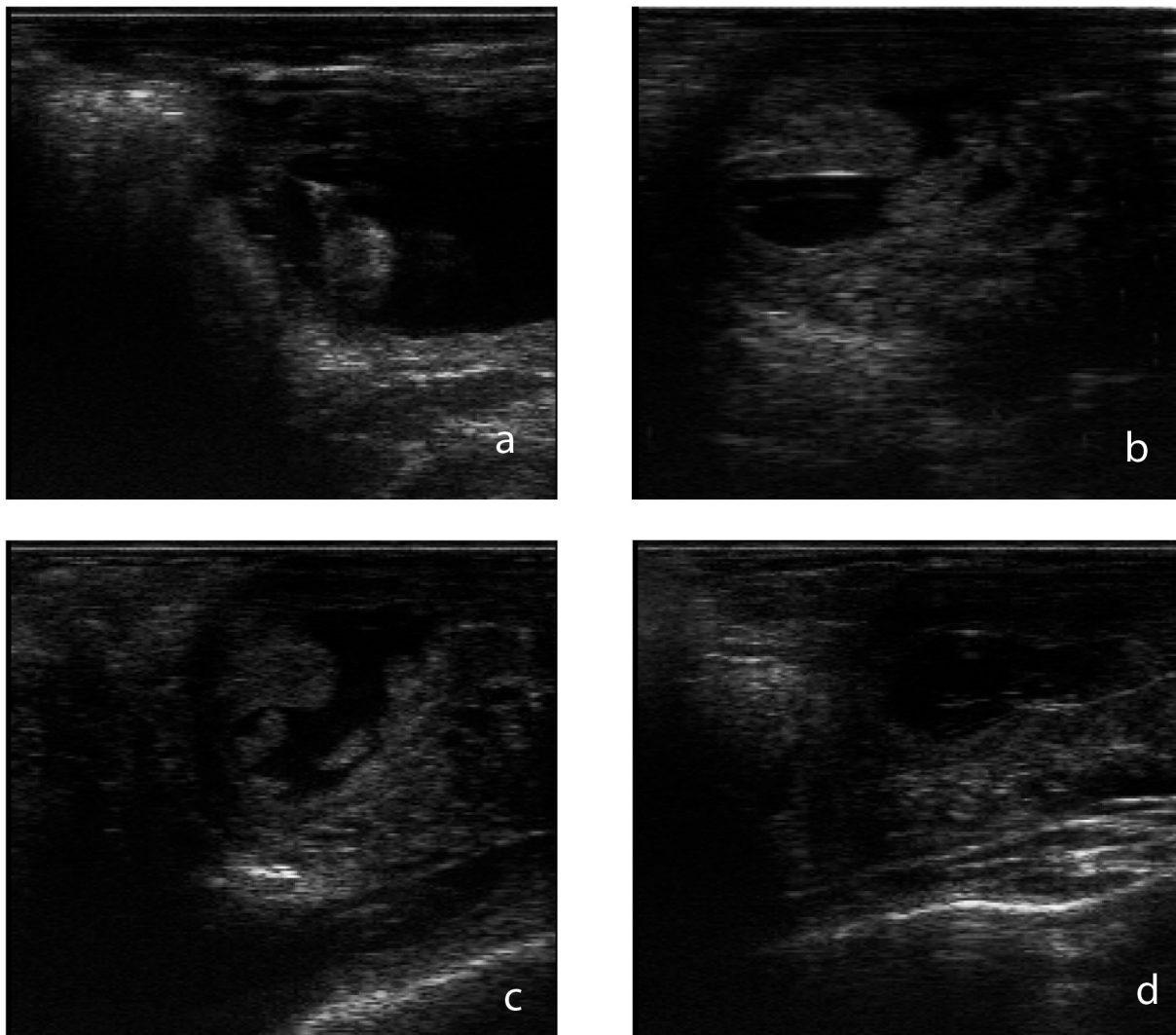
Figure 2. Echographic images of embryo mortality in a female alpaca. a — normal embryo; b — loss of shape of the embryo sac; c — partial loss of embryonic structure; d — complete loss of embryonic structure.
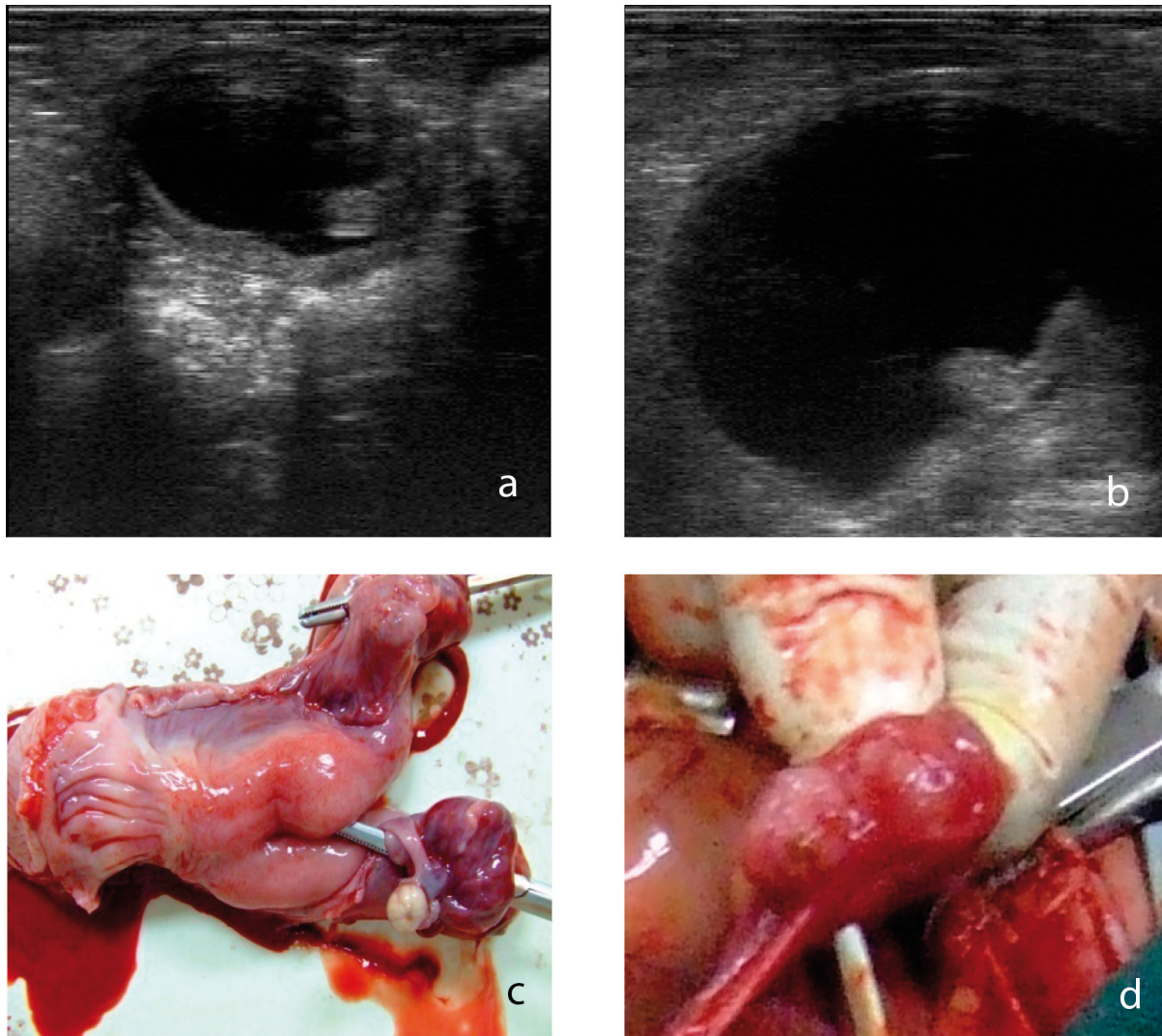
Figure 3. Reproductive organs and echographic images of an embryo death at day 69 of gestation in an alpaca
a — normal pregnancy, b — particles in suspension in foetal fluids, c — view of the uterus and ovaries immediately after ovarian hysterectomy showing the corpus luteum in the right ovary, d — view of the left ovary with presence of a large follicle.
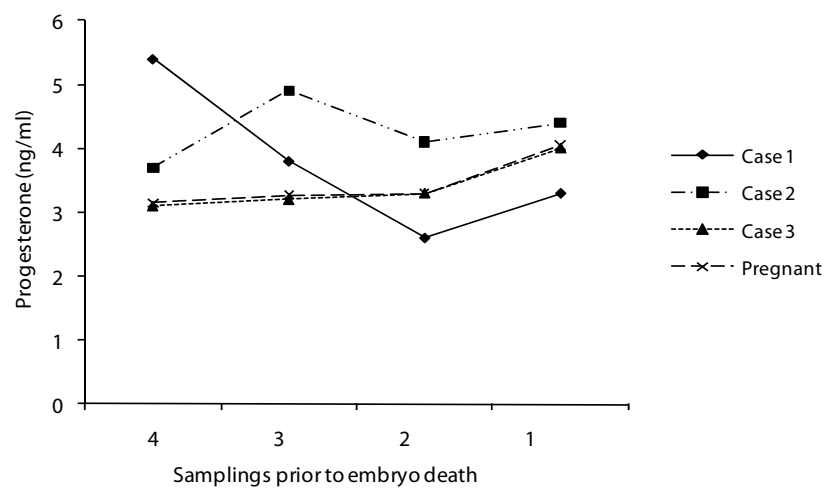
Figure 4. Progesterone levels in systemic venous blood prior to embryonic death in samples collected twice a week in three alpacas as compared with progesterone values in a pregnant alpaca in the second month of gestation.

horn, and a corpus luteum (CL) was found in each ovary. The systemic progesterone values were similar to those observed during the normal gestation and dioestrus periods (**Table 1**).

The second embryo mortality occurred on d 40 of gestation (**Figure 2**). Cheesy structures were observed in the left horn, both ovaries had a CL, and progesterone levels were reduced. Ultrasound images showed a gradual process of damaged embryonic structures. In the third case, mortality occurred around 69 d of gestation. Gestation took place in the left horn and a CL was present in the right ovary. Previous ultrasound images showed evidence of suspended particles in foetal fluid (**Figure 3**).

Serum progesterone levels were different between local (ovarian vein) and systemic circulations (jugular vein) in relation with reproductive status of animals (**Table 1**). Progesterone concentration in systemic blood was high during the four samplings prior to detection of embryonic deaths and similar to animals carrying viable embryos (**Figure 4**).

DISCUSSION

Three cases of embryonic death were observed among 16 pregnant alpacas during the first 90 d of gestation, representing a 28% embryo mortality rate. This number is lower than the 50% previously reported in the first 30 d of gestation in the classical study of Fernández-Baca et al. (1970). Only one embryo was lost in the first 30 d of gestation and the other two died in the second month of gestation (d 40 and 69). The late mortality, if usual in these animals, might result from a management problem as routine technical procedures indicated that female sexual receptivity should be teased with males two weeks and one month after mating to rebreed those accepting the males. The results indicate that females diagnosed as pregnant at the middle or final part of the breeding season will not deliver a calf as they will not have another opportunity to be bred.

The deleterious processes in the embryos are clearly shown by the ultrasound images. Autolysis in the embryo initiates after suspension of cardiac beats, which is followed by a gradual decrease of the volume of placental fluids, and finally, only membrane remnants can be seen (Ginther, 1985). Progesterone secretion by the CL was not involved in any of the embryo losses since in all three cases peripheral serum levels were higher than 1 ng/mL, and those values are considered from functional CLs (Stefanczyk-Krzymowska, 1998). Also, progesterone levels at the initial stage of gestation were similar to those reported in the literature (Aba et al., 1997; Raggi et al., 1999; Echevarría et al., 2007).

Surgery in the first case was performed during the process of embryo mortality and the progesterone concentration in the ovarian vein was similar to values obtained during the dioestrus period and those during normal gestation (**Table 1**), but quite different from the serum basal progesterone levels recorded on the d of mating and on d 13 post infertile mating (Raggi et al., 1999; Echevarría et al., 2007). Progesterone values in the ovarian vein in the other two cases were still high but much lower than in the first case as the process of embryo mortality was already complete. However, the data clearly showed that CLs were functional while embryos were dying.

Progesterone levels in the ovarian vein are directly related to ovarian progesterone secretion (Stefanczyk-Krzymowska et al., 1998), as this blood vessel originates in the ovary. On the other hand, progesterone levels in the uterine vein were similar to those in the jugular vein.

Embryo mortality is a multifactor process that needs to be elucidated in alpaca. Results from this study indicate that the CL is functional during the process of embryonic death and therefore, other factors probably related to the quality of the embryo may intervene and have to be elucidated in further studies.

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Establishment of Multiple Ovulation and Embryo Transfer (MOET) Technology for Goats in Sri Lanka

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ABSTRACT

This study was conducted to determine a suitable follicular stimulating hormone (FSH) preparation for superovulation in goats, establish techniques for embryo production and transfer in goats, and to examine the feasibility of applying such techniques in Sri Lanka. Two groups of genetically superior does were inserted with progesterone releasing intravaginal pessaries (45 mg Cronolone) on d 1 of the programme. On d 8, the does in Group 1 (n = 3) and Group 2 (n = 4) were given 2.5 mL injections of pure porcine FSH (pFSH, 20 mg/mL) or pure ovine FSH (oFSH, 0.88 mg/mL), respectively. On the same day, all animals were injected with 300 IU pregnant mare serum gonadotropin (PMSG, 500 µg/mL). Subsequent injections of 1.25 mL pFSH or oFSH were given in the morning and evening on d 9 and 10. Does were injected with 197 µg prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$, 263 µg/mL) in the morning of d 9 and vaginal pessaries were removed on the evening of d 10. On d 11, 1.25 mL of pFSH or oFSH and 1 mL of luteinising hormone releasing hormone (LHRH, 50 µg/mL) injections were given in the morning and evening, respectively. On the same day, does in oestrus were bred to two Jamnapari bucks. Seven d postoestrus, embryos were collected surgically, using embryo flushing medium. The quality of the embryos was assessed and the recovered embryos were transplanted surgically to oestrus synchronised goat recipients (n = 4/group) at 7 d post-oestrus. Following embryo transplantation, four does (Group 1, n = 1, Group 2, n = 3) were found to be pregnant by ultrasound scanning at 35 d into pregnancy. One healthy female offspring (Peradeniya Kumari) was born to Group 1. Another four goat kids were born to Group 2, while one kid died. In the same group, one abortion was reported. The results suggest that oFSH is better than pFSH for the superovulation of goats and that embryo transfer technology can be used in goats in Sri Lanka.

Key words: *goats, hormones, oestrus synchronisation, multiple ovulation, embryo transfer.*

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INTRODUCTION

Reproductive biotechnologies play a key role in improving animal reproduction. Multiple ovulation and embryo transfer (MOET) is a technique in which embryos are collected from a genetically superior (donor) animal and transplanted into another animal (recipient) for the remainder of development until term (Betteridge, 1981). It can be used to multiply genetically superior females by increasing reproductive efficiency, as an easy method to transport genetic material across the world at low cost and with minimum risk of spreading diseases, together with embryo sexing to get more female offspring from a genetically superior animal (Abeygunawardena, 2002; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004). It can also be used to conserve endangered species (Senger, 1999). With the combination of embryo splitting, the multiplication rate of the donor can be further accelerated and identical twins can be made according to the aims of the research. Embryos can be produced even from animals which have conception failures or are unable to have normal pregnancies (Noakes, 1986).

Several key steps are involved in the MOET process including synchronisation of the oestrous cycles of donor and recipient animals, superovulation of donor animals, artificial or natural insemination of the embryo donor, recovery of embryos from the donor animal, *in vitro* maintenance of quality embryos until transfer, and transfer of embryos to recipient animals.

Usually embryo transfer (ET) in goats and sheep is performed through a laparotomy under general anaesthesia and two to four embryos are simultaneously transferred to a recipient (Alexander, 2005). For successful embryo transfer, both donor and recipient animals should be in the same stage of the oestrous cycle (Senger, 1999).

There have been no studies in Sri Lanka to investigate the feasibility of ET in goats. Therefore this study was carried out with the objectives of comparing the efficacy of using pFSH and oFSH for superovulation of goats and establishing the techniques for embryo production and transfer in goats in Sri Lanka.

MATERIALS AND METHODS

Selection and Preparation of Goats for ET

Genetically superior Jamnapari donor goats were selected on the basis of their production and reproductive performance and their pedigree records. They were 3–5 y old and between 3 and 6 parities. Donors were 2–3 months from their last weaning and 5–6

months from their last kidding. Their health status was examined and anthelmintics and multivitamin injections were given. Four d after the injections 1 mL of tetanus toxoid was given to all selected goats. The animals were provided with *ad libitum* forage, 650 g of concentrates and 30 g of mineral mixture daily for two months.

Embryo recipient goats were selected on the basis of their reproductive performance and health status over the previous three years. They were treated with anthelmintics and given multivitamin injections. Four d later, 0.5 mL of tetanus toxoid was given to all recipient animals. These animals were provided with *ad libitum* forage, 400 g of concentrates and 15 g of mineral mixture daily for two months.

Two genetically superior Jamnapari bucks raised at the Veterinary Teaching Farm were selected as studs. Although semen quality was not studied, they had demonstrated good serving capacity and had successfully bred females over the previous 2.5 y. They were given forage *ad libitum*, 650 g of concentrates and 30 g of mineral mixture daily.

Superovulation of Embryo Donors

The selected does were inserted with progesterone releasing intravaginal pessaries (45 mg Cronolone, Intervet) on the morning of d 1 of the superovulation schedule. Does were divided into two groups. Does belonging to Group 1 (n = 3) and Group 2 (n = 4) were injected intramuscularly with 2.5 mL of pure pFSH; (Folltropin-V, 20 mg/mL NIH-FSH-P1, BIONICHE, Canada) and pure oFSH; (Ovagen™, 0.88 mg/mL NIADDK-oFSH-17-Standard, ICPbio Limited, New Zealand) respectively, on d 8 of the programme. In addition, 300 IU PMSG, (Folligon, Intervet International BV, Boxmeer-Holland) was given to all does on the evening of d 8. Folliculogenesis and maturation were further supported with subsequent injections of 1.25 mL pFSH or oFSH in the morning and evening of d 9 and 10 (Gonzalez-Bulnes et al., 2004). Does were injected with 197 μg of prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$, cloprostenol sodium; PGF Veyx fort, Veyx Pharma, Schwarzenborn) in the morning of d 9 and vaginal pessaries were removed in the evening of d 10. On d 11, 1.25 mL of pFSH or oFSH and 1 mL of LHRH (50 μl/mL, Depherelin Veyx Pharma, Schwarzenborn) were injected in the morning and evening, respectively. Immediately after the LHRH injection does in each group were kept separately for 48 h with a genetically superior Jamnapari buck for natural breeding. At 12-h intervals bucks were exchanged between the two groups.

Synchronisation of Embryo Recipients

On the morning of d 1 intravaginal progestegen-impregnated pessaries (Chrono-gest, which contained 45 mg flugestone acetate; Intervet), were inserted into all recipients, (eight crossbred does), and 125 μg of PGF$_{2\alpha}$ (Veyx® fort, Veyx-Pharma GmbH, Schwarzenborn) was administered intramuscularly to each animal. On d 17, vaginal pessaries were removed, and 400 IU of PMSG; (Folligon, Intervet) were given to each doe by intramuscular injection. The animals were observed for visible signs of oestrus on the following day.

Surgical Embryo Collection

Seven d after mating, embryos were collected as follows from three and four donors in Groups 1 and 2 respectively.

Intramuscular (IM) injections of xylazine 2% (0.2 mg/kg BWt) were given intramuscularly to sedate embryo donors (Bishop, 2001), and ketamine hydrochloride 10% (22 mg/kg BWt), was injected 20 min later. Once the donors were anaesthetised, they were kept on a surgical cradle in a dorsal recumbent posture. After shaving of the ventral abdominal region of the animal, the surgical site was

scrubbed alternatively with 70% isopropyl alcohol and povidone iodine solution three times each. The uterus and both ovaries were exteriorised through mid ventral laparotomy (7 cm).

After measuring the length, width and thickness of each ovary, the number of corpora lutea in each ovary were counted. The uterotubal junction of the right uterine horn was pierced with a blunt ended 18 G hypodermic needle and the tip of an embryo flushing catheter (Tom cat catheter; 3 ½ FR, 14 cm; Sovereign™, Mexico) was inserted and pushed towards the uterine horn. The same uterine horn was pierced with a small artery forcep at the level of the bifurcation and a two-way pediatric silicon elastomer coated Foley catheter (8 Ch/Fr, 3/5 mL/cc; Unomedical, Malaysia) was inserted. After inflating the cuff, the stylet of the Foley catheter was removed. Fifty mL (10 mL × 5) of embryo flushing medium (lactated Ringer's solution with one percent bovine serum albumin) was passed through the flushing catheter and with gentle tapping on the uterine horn, fluid was collected into a 100 mL beaker. The same procedure was repeated for the left horn. After removal of both catheters the incision on the uterus was sutured with 3/0 cat gut (Ethicon). After applying hydrocortisone cream the reproductive tract was repositioned in the abdominal cavity.

Depending on BWt, the required doses of intra-abdominal and intra-muscular penicillin streptomycin were administered. The peritoneum and muscle layers of the incision line were sutured using 1 USP chromic cat gut and a simple interrupted suture pattern, while the skin incision was sutured with 0.45.G nylon using a simple interrupted suture pattern. Coumaphos, propoxur and sulfanilamide containing powder (Negasunt, Bayer Polychem, India) and povidone iodine solution were applied to the site after suturing.

Evaluation of Embryos

Embryos were separated from the flushing medium just prior to the evaluation process using a wire trawl. Their quality was assessed as excellent, good and poor (IETS, 2008).

Surgical Embryo Transfer

Eight crossbred recipient does were divided into two groups and subjected to a 12 h withholding period of feed and water. Blastocyst or morulae stage embryos were transferred on the same day as described below, to four recipients in each group.

Recipient does were placed on a surgical cradle in dorsal recumbency. The lower abdominal area was shaved and disinfected using povidone iodine and 70% isopropyl alcohol. The cradle was tilted 60^0 with the head facing down. An incision of approximately 1.5 cm was made in the skin about 3 cm to the left of the midline and about 5 cm from the udder, using a no. 23 scalpel blade. A trochar and cannula were inserted into the abdominal cavity through the incision made on the skin. The trochar was removed and a laparoscope (6.5 mm diameter) was carefully inserted through the cannula into the abdomen. Another incision was made on the right side in the same manner and a Babcock forcep was introduced. The uterine horns were visualised using the laparoscope and the ovaries were carefully inspected to find the ovary with large corpora lutea (CLs).The tip of the respective horn was grasped and three–four cm exteriorised using the Babcock forcep. At the same time the embryos were loaded into the tip of a 3.5 Fr tom cat catheter with the help of an insulin syringe.

In each transfer, three embryos of excellent and good quality were loaded onto the tip of the catheter as follows: First, a small amount of embryo holding medium (Vigro holding plus, AB Technology) was aspirated into the tip of the catheter followed by an air bubble and then the embryos with medium and finally another air

bubble. The uterine horn was punctured (very close to the utero-tubular junction) using a blunt 18 G needle and the tip of the catheter was inserted into the lumen of the uterine horn and the embryos expelled. Before releasing the horn into the abdomen, the tom cat catheter was examined to confirm that all the embryos were expelled successfully. This was carried out by washing and pipetting the catheter with a small amount of embryo holding medium in a 35 mm dish under the microscope. The abdominal incisions were sutured using 1USP-chromic catgut with a far-near-near-far suture pattern. A long-acting penicillin streptomycin injection was administered. The does were monitored post-operatively for several h and released to the shed.

Pregnancy Diagnosis

Pregnancy confirmation was carried out at 35 d post ET using an ultrasound scanner attached to a 7.5 MHz linear type per rectal probe.

Data Analysis

Mean values for the parameters measured during the superovulation and embryo transfer processes were compared between groups using the Student's t-test. Differences were considered significant at P values < 0.05.

RESULTS

Donor and recipient animals showed oestrus 24–36 h after removal of sponges. The common signs of oestrus were swollen hyperemic vagina, clear colourless vaginal mucus discharge, frequent wagging of the tail and restlessness (Abeygunawardena, 2002), but the animals did not exhibit all signs at any given time.

Values for the parameters measured during the superovulation and ET processes are given in **Table 1**, while the number and quality of the embryos recovered from each group are shown in **Table 2**. Following embryo transplantation, four does were found to be pregnant. One healthy female goat kid was born to a doe in Group

1with a birth weight of 3.6 kg at full term. Another four kids with birth weights of 3.2 kg (♀), 1.8 kg (♀), 1.6 kg (♂) and 1.2 kg (♂) were born at full term to does in Group 2. The third kid of the last three kids born as a triplet died shortly after birth. In Group 2 there was one abortion.

During the first six weeks weight gains of the first kid born to Groups 1 and Group 2 were 152.3 g/d and 149.2g/d respectively.

DISCUSSION

The study described here resulted in healthy live offspring, claiming the first five kids born through ET technology in Sri Lanka.

Signs of oestrus are reported to depend on a number of factors, such as the health status of the animal, nutrition, environment, breed etc (Stephen, 1971a).

The flushed embryos were in different developmental stages such as compacted morulae, blastocyst stage and expanded blastocyst stages. This could be due to the asynchrony of ovulation and fertilisation of oocytes (Cognie et al., 2004). A few unfertilised and degenerating embryos were also found.

The responses of embryo donors to the superovulatory treatment differed — a finding explained by the fact that responses to exogenous hormones depend on several factors such as the level of nutrition, age, breed and reproductive status of the embryo donors (Gonzalez-Bulnes et al., 2004). In the present experiment the number of embryos recovered ranged from zero to nine and the number of transferable embryos varied from zero to seven. Use of recently improved gonadotrophin preparations and programmed insemination protocols in this experiment could not avoid this variability.

Low oestrogen levels produced by the granulosa cells of developing follicles exert a negative feedback on the secretion of gonadotrophin. Similarly, inhibin secreted by developing follicles also exerts a selective inhibitory action on the secretion of FSH (Greenwald and Terranova, 1988). The number of ovulations and transferable embryos following administration of commercial FSH preparations depends on the number of small antral follicles (2–3 mm) present in the ovaries. Similarly the presence of large follicles (>6 mm) at the onset of

Table 1. Size of the ovaries, number of *corpora lutea* and number of embryos produced in two groups of donors.

Parameters	Group 1	Group 2
Mean size of the ovary (cm)±SEM		
Length	2.4±0.1	2.5±0.2
Width	1.2±0.1	1.7±0.1
Thickness	1.1±0.1	1.5±0.2
Number of *corpora lutea* (range)	6–10	11–17
Mean number of *corpora lutea*	7.6±1.2	14.25±1.2
Mean number of embryos per animal	4.3±2	4.25±2

Table 2. The number and quality of embryos recovered.

Animal group	Hormone used	Number of embryos produced of given quality		
		Excellent	Good	Poor
Group 1	pFSH	2	8	3
Group 2	oFSH	4	11	2
Total		6	19	5

the superovulatory treatment exerts an inhibitory effect on the final number of transferable embryos recovered (Cognie et al., 2003).

In cycling animals, the size of ovaries correlated with the number of developed and developing follicles present. In the present study, the mean size of ovaries in Group 2 was comparatively higher than that in Group 1. Ovulations after pFSH and oFSH stimulations were 7.6 and 14.25 in Groups 1 and 2 respectively. Thus oFSH had a superior superovulatory effect in goats. However, in this study there was no correlation between the number of ovulations and number of embryos recovered, especially in Group 2. In this group there was one blind fallopian tube in the tract of two animals; but they were having more ovulations. This could be the reason for the lower embryo recovery in such animals.

The exchange of bucks between the groups provided equal opportunities to animals in both groups and also increases the fertility of embryo donors.

One abortion was reported in this study. Viability of the foetus or the embryo depends on many factors including a functional corpus luteum, alterations in the preovulatory follicles and level of ovarian abnormalities (Gonzalez-Bulnes et al., 2004). From the five kids that resulted from the experiment, one died shortly (within 20 min) after parturition. The cause of the death may have been hypoxia, due to placental detachment. Delayed kidding may be due to foetal mal positioning, calcium deficiency, uterine inertia, abdominal muscle fatigue and nutritional deficiencies (Stephen, 1971b).

The last triplet of kids had relatively low birth weights, likely due to the high nutritional demand of all three foetuses from the same maternal tissues throughout their gestation period.

In this study all procedures were conducted under conditions similar to those in field situations using portable instruments, so that they could be repeated under farm conditions.

At present there is good potential for using MOET to establish elite herds in selected goat breeding farms. This would enable them to provide improved breeding animals, which are currently in short supply, to smallholder farmers.

CONCLUSIONS

Compared with pFSH, oFSH was found to be better for superovulation in goats. It is feasible to produce viable offspring of goats using ET in Sri Lanka, but further studies are needed to optimise the procedures and reduce the costs.

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Is Embryo Transfer a Useful Technique for Small Community Farmers?

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ABSTRACT

Four main aspects of embryo technology are dealt with in this paper. The first analyses the reasons for the poor selection of recipients for embryo transfer, the second relates to inaccurate evaluation of embryos at least under tropical conditions, the third proposes alternative methods to evaluate embryos for selection and freezing, and the fourth analyses the feasibility of establishing this technique as a biotechnology approach for improving production in small community tropical farms.

Key words: *embryo transfer, embryo assessment, freezing, small farmers, economics.*

INTRODUCTION

Several researchers have provided sufficient evidence that the best crossbreeding programme to produce milk in the tropics is the direct cross between *Bos taurus* and *Bos indicus* (F1). The problem arises when the farmer faces the challenge of breeding the crossbred animal. If the choice is to cross with *Bos taurus* the resulting product is quite vulnerable to the harsh environmental conditions in the tropics. If, on the other hand, the selection is to sire with *Bos indicus*, then the offspring will be deficient in milk production (Madalena, 1993). Another alternative is to transfer F1 embryos to F1 dams, thereby avoiding the hazards of crossbreeding (Cunningham, 1989). Although the technique of embryo transfer (ET) has been available for many years, at least under tropical conditions there are several pitfalls such as the inadequate selection of recipients, the production and evaluation of embryos and finally, the economic feasibility of the technique itself.

Selection of Recipients for Embryo Transfer

These are usually animals displaying spontaneous oestrus or treated with hormones to synchronise this event. The shortcomings of both these methods have been described by Montiel et al. (2006). In short, the use of spontaneous oestrus is time-consuming and inaccurate (for review see Galina and Orihuela, 2007). On the other hand, when using synchronised oestrus the response with an ensuing ovulation can fail in as many as 30% of cases if the animals selected are not in reasonable body condition (Diaz et al., 2002). Moreover, if the drug used for oestrous synchronisation contains oestrogens, the response of animals displaying overt signs of oestrus without an ovulation can increase by almost two-fold (Solano et al., 2000; Velásquez,

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2004). Hence, the selection of recipients displaying oestrus but with the adequate formation of a corpus luteum can be time consuming and at times frustrating (Montiel et al., 2006).

Due to the above, embryo transfer programmes in small community farms can be tricky because the selection of recipients is restricted to a few animals in the herd and the distance between farms can pose a serious threat to success. Thus, just because of this constraint, government programmes have ceased to function as the resources necessary to visit farms distant apart are limited.

Embryo Assessment

The main components of successful embryo production are: the quality of the superovulatory response in the donor cow, the ability of the individual to recover as many embryos as possible, and the skills of the technician in judging the quality of the embryos destined for freezing. In relation to the first, figures for embryo production can vary enormously, although some groups demand that the number of good quality embryos cannot be less than eight (Baruselli et al., 2006). However, others have not been so successful (Barros and Nogueira, 2001; Chebel et al., 2008). In general, the superovulatory response can be directly related to the follicular dynamics at the moment of treatment (Bo et al., 2003). Few studies have addressed this issue although it has become apparent that animals undernourished do produce follicles of lesser dimensions and compromised fertility when compared with their well fed peers (Oliveira et al., 2002). In studies where follicular dynamics were characterised in postpartum and barren cows, it was evident that the diameter of the largest follicle can be affected by the stage of the postpartum period or the time of the year when the study was undertaken (Molina, 2000). In various experiments (Molina, 2000; Montiel et al., 2006; Alarcón 2008), the superovulatory response judged by the number of corpora lutea formed was always above nine, but the number of good quality embryos hardly surpassed five.

The recovery of embryos can be difficult especially as it has been reported that almost 30% of the donor cows have curved cervices increasing the difficulties in negotiating the catheter to flush the uterus properly (González et al.,1983). Hernández (1988) showed that even when cervices were dissected, the degree of torsion was so great that it became virtually impossible to pass the catheter even under postmortem conditions.

Another issue demanding attention for future research is the ability of the clinician to accurately determine the health of the divided embryo by light microscopy in *Bos indicus* cattle. In an early study, Aguilar et al. (2002) showed that inaccuracies in the judgment of embryos can be as high as 30%. This observation was confirmed using other diagnostic criteria (López-Damián et al., 2008; Gutiérrez, 2009; Godínez, 2009). Moreover, Marquez et al. (2005) showed, also

in Bos indicus females, that the number of healthy embryos evaluated by their resistance to freezing and their degree of apoptosis, was affected if the embryos were produced in the spring when compared with the autumn even using the same donor cows.

Methods to Evaluate Embryos for Selection and Freezing

Marquez-Alvaredo et al. (2004) studied embryos kept frozen for various periods of time and reported that these structures with shorter storage time, presented a lower number of dead cells evaluated by apoptosis compared with embryos with a longer storage. The authors concluded that embryos produced on an industrial scale have the potential disadvantage of being subjected to a variety of assessment criteria when they are selected for freezing. This observation merits further research.

  There is agreement between investigators that embryo assessment is an important source of error (Lindner and Wright, 1983). This observation has been recently confirmed (Gutierrez, 2009). In fact using invasive methods to judge embryo soundness, these researchers demonstrated that cells classified by experienced clinicians as viable, are in fact defective. Another potential source of error in ET relates to embryos which are routinely classified by stereoscopic microscopy before freezing and the quality of the embryo is no longer reassessed. Considering that the most important point in the ET is the grading of the embryos, this practice can lead to placement of cells in cows with little possibility of rendering a pregnancy as they were of mediocre quality from the start. It is also important to consider the handling and care of the embryos when they are stored, because both are critical for their viability. In an effort to reduce this shortcoming, Contreras et al. (2008) found that after culturing fresh embryos for up to 8 h, good and fair quality embryos did not undergo major detrimental changes in development even after 7 h of incubation, whereas poor quality embryos experienced changes as early as 2 h. Good quality embryos invariably had fewer numbers of apoptotic cells than those of fair and poor quality suggesting that embryo culture can be a useful method to assess viability and to confirm the quality of recently collected embryos. These results were recently confirmed by Godínez (2009) in fresh embryos, but not in cells previously frozen. Her results suggest that the culture medium can be toxic to embryos expanding after thawing. Further research is required to elucidate the reasons for these differences.

Economical Feasibility of ET among Small Community Farmers

Government organisations in developing countries have launched initiatives to popularise the evident benefits of ET, particularly in enterprises not bigger than 50 cows. These programmes have experienced a high degree of acceptance, especially those with a substantial subsidy. However, when the programme terminates, it will invariably have proven not to be sustainable for the farmers themselves; thus disappointment is the natural outcome (Molina, 2003; Chávez, 2008). In a recent study (Alarcón, 2008) estimated the cost of preparing the donor and recovering embryos was about US$600. The average number of embryos recovered was 3.8. Taking into consideration the cost of gestation, calculated as the percentage of animals pregnant (27%), the cost for preparing the donor, the technique of embryo transfer and the cost of producing the embryo itself, the overall cost per gestation was US$1320. Considering a 50:50 ratio of males:females born, the cost for a replacement heifer was US$2 640 — surpassing by far the commercial cost of a crossbred heifer (approximately US$900 dollars).

CONCLUSIONS

Considering the difficulties in distributing F1 embryos among farmers in small enterprises, the cost of production and the low success rate found in terms of fertility, for the time being it does not seem profitable for farmers themselves to sustain the costs of an ET programme. Government organisations would therefore need to play a more active and systematic role to ensure that the costs inherent in ET technology are reduced.

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The Effect of Management Practices on Prevalence of Mastitis in Large Scale Dairy Farms

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ABSTRACT

A study was conducted to investigate the impact of management practices on the udder health status of dairy cows in Thuringia-Germany. Forty-eight dairy farms were randomly selected and 64 542 milk samples from 10 741 dairy cows were collected and subjected to bacteriological investigation. The prevalence of the infection was 27.57% of the quarters, and 49.66% of the composite milk samples. *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) were the most frequently isolated contagious pathogens with udder and quarter prevalences of 28.7% and 35.5% and 26.6% and 32.7% respectively. On the other hand, *Streptococcus daysgalactia* and esculin-positive streptococci (environmental pathogens), had prevalences in udder and quarter samples of 12.9% and 13.9% and 9% and 10.6% respectively. Incidence rates were 32.8% in small herds and 31% in large ones. Housing and milking systems, feeding and udder cleaning methods significantly influenced the mean incidence rate of mastitis. Ignorance of inter-milking sanitisation resulted in a higher incidence rate (33.5%), which was lowered by practising sanitary measures (31.5%). Application of teat dipping reduced the incidence of mastitis to 32.3%, whereas, the non-use of teat dipping resulted in an incidence rate of 33%.

Key words: *dairy farms, mastitis, management, prevalence, sanitisation.*

INTRODUCTION

In recent years the demand for liquid milk has increased tremendously worldwide due to increased population growth and incomes. In most countries, dairy cattle breeding programmes are directed toward milk production traits. Although these traits are of primary economic importance, functional traits such as longevity, fertility and udder health are of increasing interest to producers to improve herd profitability. Mastitis is defined as an infection of the udder, caused by bacteria entering the quarter through the teat end (Rodenburg, 1990). Several researchers (Wendt et al., 1994; Smith and Hogan 1995; Kalmus et al., 2006) concluded that mastitis-causing organisms can be classified into two main groups: contagious pathogens which spread by means of hands and milking units and include *S. aureus, St. agalactiae,* and *Mycoplasma*; and environmental organisms which live in the cow's environment and are always present and include *E.*

coli, St. dysgalactiae, St. ubris. Hogan and Smith (1987) stated that the percentage of quarters infected with environmental streptococci is low and seldom exceeds 10% of quarters. Smith et al. (2000) stated that small herds reported more cows being removed for mastitis than high medium and low medium herd size. The National Mastitis Council's fact sheet (1997) states that housed cows are at greater risk from environmental mastitis than cows on pasture. Also, post milking teat barrier dips reduce new coliform intra-mammary infection but their efficacy against the environmental streptococci and contagious pathogens appears to be lower than that of germicidal preparations. It has been also shown that back flushing of the milking unit does not control environmental mastitis. Additionally, malfunctioning milking machines which result in frequent liner slips and teat impacts can increase cases of environmental mastitis. Washburn et al. (2002) compared seasonally calved Holstein and Jersey cows in confinement and on pasture systems and found that cows in confinement had 1.8 times more cases of clinical mastitis and eight times the culling rate for mastitis than did cows on pasture. Radostits et al. (1994) summarised the control measures of mastitis among which pre-milking udder hygiene, post-milking teat dipping and environmental control during the dry and calving periods are most important.

Each of these control measures is aimed at the management of specific pathogen types. Pankey (1989), Boddie et al. (1993) and Malinowski (2000) concluded that pre-milking udder hygiene and teat dipping are aimed at reducing infections mainly caused by contagious pathogens and preventing new infections and to a lesser extent at preventing infections that might be caused by environmental pathogens. Sargeant et al. (2001) claimed that producing high quality milk requires effective udder health programmes at the herd level. Management practices at the time of dry-off and during the dry period are essential in this respect. Peeler et al. (2000) found that the incidence of mastitis increased when milking cows were housed in a straw yard, while Oliver et al. (2001) demonstrated that pre- and post-milking teat disinfections with phenolic combination were significantly more effective in preventing new intra-mammary infection than was post-milking teat disinfections only. They also added that pre-milking teat disinfections with phenolic combination in association with good udder preparation and post-milking teat disinfections can further reduce the occurrence of new intra-mammary infection by numerous mastitis pathogens during lactation. Saloniemi and Kulkas (2001) in describing mastitis control in Finland, recommended post-milking teat dipping as a control tool in herds with contagious udder pathogen problem.

MATERIALS AND METHODS

Milk samples from 10 742 dairy cows in 48 large scale dairy farms in the state of Thuringia-Germany that were calving between June

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1998 and April 2000 were used in the study. Milk samples were subjected to bacteriological investigation and a questionnaire for the collection of management data was prepared which included housing system, milking system, udder cleaning methods, inter-milking sanitation of the milking units and post-milking teat dipping. Bacteriological and questionnaire data were merged into one data set by means of a statistical program using the SAS package (SAS, 1996). Data were analysed using the frequency procedure of SAS (SAS, 1996) and results were presented as contingency tables.

RESULTS

Figure 1 displays the frequencies of the bacterial types that were found in the udder quarter and composite milk samples. The total positive findings were estimated to be 15 701 and 3 765 respectively, which represented 27.6% and 49.7% of the total samples collected from each site. *Staphylococcus aureus* and CNS were the most frequently isolated pathogens from the udder quarter samples and composite milk samples (35.5% and 28.7% and 32.7% and 26.6% respectively). However, *Streptococcus daysgalactiae* and EPS infections were more frequent in the composite milk samples than in the udder quarter samples (13.9% and 12.9% vs. 10.6% and 9.0% respectively).

The study also indicated that the infection rate was influenced by the herd size (**Table 1**), farms with large herd size having a significantly lower infection rate (31%) compared with those having small herd size (33%). It was also found that infection rate decreased steadily as the number of animals increased.

Infection rate was found to vary between housing systems. Animals housed in either slat or plain floor loose housing barns had higher infection rates (32% and 31.8% respectively) than animals kept in barns other than loose housing (30.5%). Milking systems also influenced mean infection rate, being significantly higher (33.5%) in animals milked in pipe system units than those milked in either carrousel or milking parlour units (32.5% and 32.3% respectively).

Results revealed a significant statistical effect of feeding method on infection rate, with rates being higher in farms using both mobile and stationary methods of feeding (32.5%) compared with farms using stationary method of feeding (31%).

Methods of udder cleaning before milking had significant effects on infection rate. Farms which used moist udder cleaning had slightly higher mean infection rate (32.5%) than those that were practising dry udder cleaning (32.3%). **Table 2** shows the effect of the sanitisation methods used in the milking units on infection rate. Infection rate was highest in farms that ignored sanitisation between milking (33.5%); this was nearly the same as in the farms that were

Table 1. Infection rate of mastitis as influenced by herd size.

Class of the herd size	Number of cows	Infection rate (%)
Small	< 200	33.0
Medium small	200–400	32.0
Medium	401–600	31.8
Medium large	601–800	31.3
Large	> 800	31.0

Table 2. Infection rate of mastitis as influenced by inter-milking sanitisation method.

Sanitisation methods between milking	Infection rate (%)
Back flushing	33.3
Air wash	32.0
Bath (Tub)	31.8
Spraying	32.0
Other	31.5
Not used	33.5
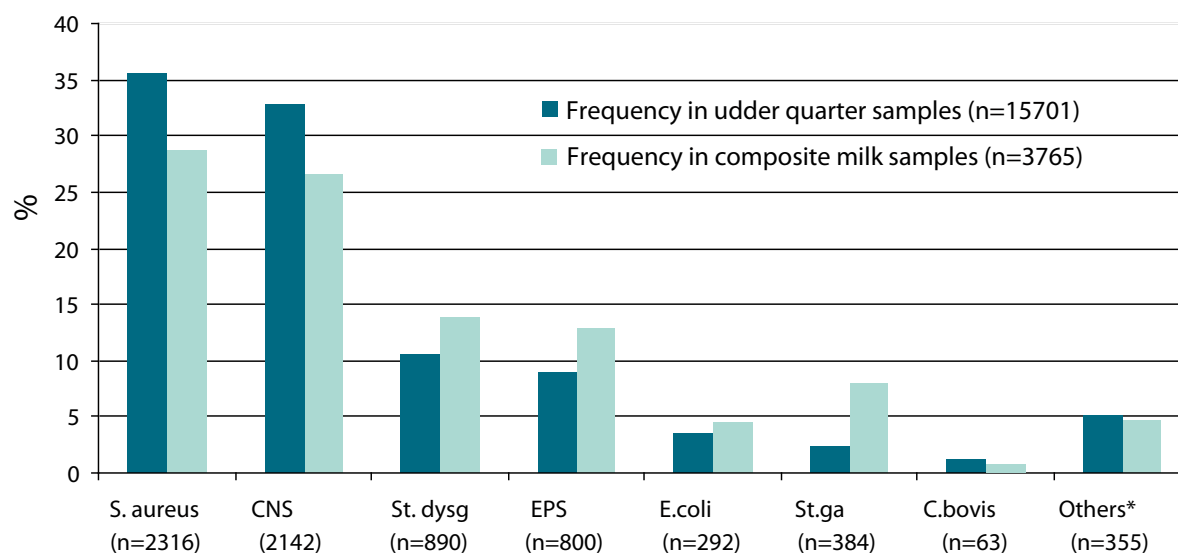
* — *Pseudomonas aeruginosa, Actinomyces pyogenes,* spore forming bacteria, yeast etc.

Figure 1. Frequency of individual bacterial types in udder quarter and composite milk samples.

using back flushing (33.3%). The use of a combination of methods reduced the infection rate to 31.5%; this is significantly different from the aforementioned means. The use of air wash and spraying resulted in infection rates that did not differ (32%), whereas the use of the bath lead to an intermediate infection rate (31.8%).

Teat disinfection also influenced the degree of the infection, teat dipping reducing the mean infection rate to 32.3% compared with 33% when no teat dipping was employed.

DISCUSSION

Mastitis control is a never-ending battle for dairymen with many individual and interacting factors involved in causing problems. The present study was based on 64 542 randomly collected foremilk sample (56 960 were samples from quarters and 7 582 were composite milk samples). The most frequently isolated pathogens from both types of samples were *Staphylococcus aureus* and CNS — a finding consistent with those of Trinidad et al. (1990), Nickerson et al. (1995) and Waage et al. (1999). In small farms, infection rate was highest, and decreased gradually to reach the lowest value in the large farms. These results are in agreement with Wilesmith et al. (1986), who showed that the incidence of mastitis declined with increasing herd size. Dego and Tareke (2003) found that the prevalence of mastitis was significantly higher in Holstein-Friesian than in indigenous Zebu cows and in non-lactating than in lactating cows.

Farm management and hygienic factors are considered to be among the main risk factors, as they predispose the animal to intra-mammary infection. The study investigated the influences on intra-mammary infection of housing systems, milking techniques, feeding methods, udder cleaning methods before milking, inter-milking sanitisation methods as well as post-milking teat disinfection. Cows housed in muddy lots or pastures are obviously at a high risk for pathogen contact via organic bedding materials or dirty stalls. Mean infection rate was significantly higher in animals kept on plain floors and in loose stalls, followed by animals kept in slat floored loose housing where animals had the same infection rate as those kept in other stall types. Among the other housing types is the tie-stall barn in which animals are always under threat from pathogens as the stanchion limits animal movement and subjects the teat to injury. In loose barns, there is also the problem of lying on rubber floors or straw bedding. Well maintained and loose bedded stalls and well drained dry lots minimise possible contamination of the teat ends from inter-mammary infection causing pathogens compared with animals managed in pasture. This conclusion is supported by Peeler et al. (2000) who found that the incidence of mastitis increased in milking cows housed in straw yards, as well as those standing in a yard after milking. Also by Rodenburg (1990) who showed that stalls that were too small subjected animals to injury; in free-stall barns cows are less likely to lie in dirty and such barns are always of adequate size.

Milking techniques are also considered as factors that can affect the udder health status of the cow. Milking units are the primary means of transferring contagious bacteria from cow to cow. In infected herds there will be relocation of bacteria from infected cows to non-infected cows by the milking unit and this allows mastitis spread. Pipeline milked animals had a significantly higher frequency of pathogens (35.5%) and consequently higher mean infection rate. If pipelines are not correctly and regularly cleaned and rinsed with plenty of water this will lead to bacterial lodgment and raise the problem of inter-pipe pathogen transmission. Animals milked in carrousel units had higher mean infection rates than those milked in milking parlor. Better cleaning and disinfection of the milking unit always leads to reduce the effect of the pathogens. This difference is again of a managerial nature as superior management of the milking unit is assumed

to improve udder health status of milking herds. On the other hand, faulty management (besides sampling error that should always be taken into consideration), will exacerbate the condition.

Farms using both mobile and stationary methods of feeding were found to have a higher frequency of pathogens than those using only a stationary method or mobile method of feeding. These consequences are believed to be slightly dependant on the kind of feeding system, but to a great extent on the nature of the feeding and feeding equipment. This is in addition to how well such equipment is cleaned after feeding in order to prevent carry-over of contaminants whether contagious that can be transmitted through hands or environmental which live in a suitable environment created by faulty management processes.

To achieve an optimum level and quality of production, it is of paramount importance to clean the udder of the cow before commencing the milking process. In this study two types of udder cleaning were routinely performed, moist and dry. The mean infection rate in animals whose udders were cleaned by moist means was significantly higher than in animals whose udders were cleaned by dry cleaning. This difference could be attributed to the fact that moist cleaning can predispose the animal to intra-mammary infection, and since the intra-mammary infection causes pathogens to enter the udder through the teat opening, milking wet teats increases considerably the chance of forcing bacteria into the quarter. Also when a disposable towel is used to dry the teat of more than a single cow, this will overwhelm the condition and allow bacteria to be transmitted between cows. Higher frequencies of pathogens resulted in higher mean infection rates of the animals milked in milking units that were not subjected to sanitisation compared with in farms practising inter-milking sanitisation. These findings emphasise the importance of sanitisation as a routine management practice to control or reduce intra-mammary infection.

From analysis of the sanitisation methods examined, it can be concluded that a combination of one or more methods were effective in reducing the mean infection rate. Spraying was otherwise the most effective method in reducing contagious pathogens, while bathing was effective in reducing environmental pathogens. Back flushing was not effective in reducing infection rate with either contagious or environmental pathogens, a finding that was also reported by the US-National Mastitis Council's fact sheet (1997). Of the other hygienic measures adopted by the dairy farms inspected, teat dipping reduced the frequency of pathogens (37.3% infection in farms practising dipping compared with 40% in those that did not). Natzke (1981), Pankey (1989), Boddie et al. (1993), Radostits et al. (1994), and Malinowski (2000) all concluded that teat dipping reduced infections mainly caused by contagious pathogens but also prevented new infections and to a lesser extent infections caused by environmental pathogens. Oliver et al. (2001) demonstrated that pre-and post-milking teat disinfection with phenolic combination was significantly more effective in preventing new intra-mammary infections than post-milking teat disinfections only.

CONCLUSIONS

The most frequently isolated pathogens were *Staphylococcus aureus* and CNS, which led to significantly higher infection rates in the farms studied. Herd size affected the degree of infection in that small herd sizes were more at risk from udder infections than large herds. The use of hygienic measures are of utmost importance in reducing infection rates.

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Foundations, Fallacies, and Assumptions of Science for Livestock in Development

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ABSTRACT

Molecular genetics is a new scientific discipline offering the technology to transfer exotic genes into livestock species. Scientific and business interests aim to apply this technology in the near future to make genetically modified (GM) livestock for the food chain. In Europe there is a strong move by citizens against milk and meat products from GM livestock. The possibility of using the technology on livestock in the developing world is under consideration as advocates claim that it would be a major contributor to world food security. This paper presents the opposite view. There are several sets of reasons against using this new technology at this time that are explored here. First, scientific knowledge of the mammalian genome is inadequate and a vast amount of research is needed before success will be ensured without negative consequences for humans and animals. Second, livestock are an essential resource for survival of billions of rural poor in the developing world and they should not be exposed to risk. Third, ethical considerations are not evident but are essential because the plans are so radical and affect public interest at many levels. Scientists today show lack of wisdom in failing to see the consequences of using their limited knowledge. Reasons for this absence of wisdom are explored in a brief review of the historic development of science. Livestock scientists need to learn lessons from the sagas of GM crops and mad cow disease (BSE). Other ways to empower the poor to increase food security are described. Scientists are urged to continue research and to seek a moratorium against GM livestock being used for food until objective and tested results enable stakeholders to decide.

INTRODUCTION

This paper covers the subject matter by dealing with four inter-related topics: the role of scientists in society, the present world situation, genetics and revisionist geneticists, and molecular biology, livestock and the poor.

THE ROLE OF SCIENTISTS IN SOCIETY

The West versus the Rest

Measured by scientific, economic and military power, the West is the most advanced society in human history. In these terms, other societies in the world today have less clout; but they are nevertheless long-established and sustainable with their own cultures, lifestyles, values and world views. In these economically simpler rural communities several billion people are still dependent upon livestock as a primary resource for life - just as in the West not so long ago. The agenda of this Symposium examines the transfer of molecular biology technology and products to those societies for use on the genomes of livestock. While offering total support to the objectives of improved livestock production and health, enhanced food security and alleviation of poverty, this paper raises some serious reservations on scientific, socio-economic and ethical grounds about using molecular biology techniques for genetic manipulation of livestock in developing countries at this early stage of knowledge. The paper advocates caution alongside the exciting potentials most of which remain exploratory, untested and speculative. Real uncertainty lies in contemplating action programmes in developing countries for these novel molecular techniques which are not accepted by many in the West. For ten years, more than 60% of EU citizens have consistently rejected GM foods (EuroBarometer website) and, in 2008 through their parliamentary representatives, EU citizens overwhelmingly rejected milk and meat products from cloned livestock. These reservations call for deeper examination of using such technologies and their products in rural societies where livestock are the major foundation of life and of society.

There is a vast difference between research and application of molecular biology. This paper fully supports research and affirms that this Symposium will advance knowledge of the livestock genomes thereby contributing positively to scientific research. But, logically, there is something incongruous about importing unproven technology to change extensive livestock systems of Africa, Asia and Latin America when intensive farming systems are themselves proving unsustainable in the developed West. Wisdom demands answers to some deep questions in these circumstances. The question is not about researching the potentials; it is about use in the foreseeable future. Responsible use must be built upon sound scientific knowledge which, at present, is sparse on the molecular universe of the mammalian genome.

Effective use of scientific knowledge must be accompanied by a deep understanding of human values upon which civilisation has been built. This duet of scientific knowledge and social wisdom goes beyond the protocols needed in a research laboratory. Human societies in rural settings, though simple and based upon livestock, have proved to be sustainable over millennia and deserve respect. Scientific knowledge and social wisdom must proceed together to understand the local and global matrices of human affairs. This has always been the posture of good science — until recently. Today the newly emerged worldview of many scientists sets caution aside and moves into immediate use of the new molecular genetic techniques in food. Scientists who question this position are often seen by their peers as heretics.

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Why Address Genetic Manipulation of Livestock at this Time?

This paper addresses the foundations, fallacies and assumptions of science in the context of genetic manipulation of the livestock genome to produce transgenic animals. Why? The reason is that society today is poised on the brink of using these techniques to produce human food. So far as publicly known, no milk and meat products from cloned or transgenic animals have yet reached the market. But the scene is being actively prepared, as shown by the following facts:

- clones and transgenic animals have been produced in all the major livestock species since Dolly, the cloned sheep, was born in 1997;
- in 2006, the USA Food and Drug Administration (FDA) approved milk and meat from cloned cattle, pigs and goats for the human food chain without labelling;
- large biotechnology companies are already engaged in promoting transgenic livestock as a means of improving animals and their products for human consumption (Biotechnology Industry Organization, 2008);
- some of these companies already hold patents on transgenic livestock in the USA and the EU;
  further, this International Symposium raises the possibility of using these techniques in developing countries.

This paper opposes the use of transgenic livestock in practical farming and in the food chain because release of such animals would irreversibly change the agro-bioresources and the environment; also these manipulated resources cannot easily later be withdrawn. These changes would be particularly hazardous in developing countries. This paper does not oppose the use of molecular biology in the field of animal health where new products for old animal disease problems are being generated for diagnosis, prevention and treatment. These biotechnology products can be researched and produced within controlled conditions. The products come from genetically modified organisms (GMOs) but normally do not include tissues containing DNA itself. Further, use is targeted and limited to specific animals and the choice rests with the owner of the livestock.

Wisdom and Knowledge

As scientists we are tempted to think that the scientific method has such a firm foundation that it can answer all the issues of life. After all, science has yielded phenomenal progress in understanding the material world and by improving the quality of life. We must beware of confusing the objective scientific method with scientists who are human and fallible. In 2008, at a European conference, a top animal scientist was describing and advocating immediate use of cloned livestock for food; when asked about consumer resistance he said it was not an issue as "scientists think: others feel". That is a dangerous and arrogant world view that elevates scientists to an elite position as decision-makers for society because they 'know'. Doubtless within a narrow field, one can be rational, objective and super-intelligent in processing knowledge in the abstract. Reality, however, is much more complex. The application of knowledge requires wisdom, balanced judgement and concern for the larger consequences on the whole community of life today and in the future. The 2008 global banking and financial collapse was brought about by a small group of professionals who used highly rational and thought-intensive processes to create complex financial instruments — but the values they used were those of self-interest. This scenario clearly demonstrates the dangers of the concentration of power in the absence of basic human values. Those who neglect the values that build community

lack wisdom. They cut off the branch that supports them. We violate community at our peril.

Scientists have an important role in society: basically, to serve the interests of all. Unfortunately some scientists now misunderstand their role. Rationality and reductionism are key intellectual components of the scientific method for successful study of the material universe. But the new knowledge they yield must be linked with wisdom on how such knowledge will be used. To apply a narrow scientific view to life as a whole raises the question of how knowledge differs from wisdom. Has humanity the ability and willingness to use its great intellectual resources to sustain life? This question lies close to the heart of whether emerging molecular technologies that are poorly understood should be used on livestock in simpler cultures with historically different values and traditions from the West. What is the difference between knowledge and wisdom? The simple yet profound answer is this:

Knowledge = power; knowledge + wisdom = sustainable life.

The difference between knowledge and wisdom is also highlighted by the two possible responses of scientists to any new discovery: "We can do it — so let's do it now"; or "We can do it — why do it now?"

The question of knowledge and wisdom is illuminated by a brief look at the origins of Western science that emphasises their different but complementary roles in civilisation. Modern science grew from the 15th century onwards shaped by the great European movements of the Renaissance, Reformation and Enlightenment. In the preceding European Ages, the common world view included positive as well as negative elements. Negatively, society in general was ignorant of the composition and function of much of the material world beyond the practical needs of living. Life contained much mystery. Lack of knowledge fostered superstition, for example, by attaching supernatural powers to material objects like trees, rocks, rivers, wind, fire and some animals, etc. Nevertheless, those simpler societies had many positive aspects — similar to many rural societies in the developing world today.

People living in poorer periods and countries know that community is essential for survival and for hope of a better life. In Early Medieval Europe prior to the Enlightenment, as in all sustainable societies, positive values and standards of social behaviour were basic with sanctions against anti-social conduct. Such communities lived within the boundaries set not only by natural resources and seasons, but they also cherished values that protect and sustain life. Individuality was valued, even encouraged, but was subject to the overall priority of living together. Thus, although simpler societies lacked the fuller knowledge of the physical universe brought later by science, they practised wisdom by sharing values that went deeper than material prosperity and individual success.

Before the Enlightenment, most people held a single unified worldview that included two distinct components:

- knowledge: facts about the material world based upon life experiences such as farming, weaving, building, cooking, health, etc;
- wisdom: using knowledge and resources for a sustainable life; being conscious of right and wrong and good and evil; treasuring human values that define good behaviour; being committed to quality of life for all in society over successive generations.

In that earlier society, values were learned by cultural osmosis while skills were acquired by apprenticeship working alongside experienced workers. The Enlightenment ushered in reductionism and education for professional disciplines in special institutions separate from the workplace. In the Pre-Enlightenment Age, the common worldview was illuminated by Christianity which was the historic

base of European beliefs, morals and values. The moral foundation of this world view, not always practised but accepted throughout society, was the teaching of Jesus to treat others as you wish them to treat you and not doing to others what you wish them not to do to you. The teaching style of Jesus emphasises the unity of knowledge and wisdom in that worldview, for his profound moral teaching was not abstract philosophy but was built upon practical examples from the routines of life that everyone knew.

The Enlightenment and the Birth of Modern Science

The Enlightenment challenged the Medieval unified worldview. A major change was formulated by René Descartes (1596–1650) that is particularly significant for scientists today, namely 'dualism'. In simple terms dualism means separation of what science can know from other ways of knowing. Science focuses upon the material world that is susceptible to analysis and scrutiny by objective debate. Descartes put all other components of knowing into a second category that he said is unquantifiable, subjective, and conjectural, based upon belief and sometimes invalid in some components. This dualist view of reality was new. Before Descartes, people saw a unity of the material and the spiritual. Knowledge, wisdom and superstition were mixed. But dualism separated knowledge of the physical world from the transcendent - and modern science was born.

Science slowly revealed the facts about the material world, showed how things worked and how they could be changed to improve life. This process eliminated superstition about matters that had not previously been understood. Nevertheless people and communities continued to hold and practise values and beliefs. The scientific revolution is often considered to have started formally with Sir Francis Bacon (1561–1626) who was also the inspiration for the founding of the Royal Society in England. He was an outstanding scientist and philosopher who emphasised the inductive method and recognised both categories of knowing; but he commented that "scientists have nothing to say about values". In the early centuries after the birth of modern science, wisdom about life and scientific knowledge were identified as separate but real partners in the common worldview. Unfortunately in recent decades there has been a tendency to elevate scientific knowledge above wisdom, giving rise to a posture which considers that only scientific facts matter while values can be discarded along with superstition. Some scientists today feel that scientific knowledge is the only type of information that counts. This attitude can lead to the arrogance that scientists 'think' whereas others only 'feel', thus equating feelings with superstition. This posture is both new and dangerous. E. O. Wilson (1998), the distinguished contemporary Harvard biologist described the position well: "We are drowning in information while starving for wisdom".

The early European scientists made two major systemic contributions to civilisation. They defined the foundations of the scientific method which include reductionism, hypothesis, testing, and replication. These bulwarks of modern scientific knowledge were predicated upon the assumptions that everything has been made by God who is neither chaotic nor capricious and that the material world reflects his consistent character. The second major contribution of the early scientists was wisdom to use science in a moral and ethical way for the welfare of all society. In general, they were men and women of integrity, transparency, honesty, modesty and objectivity. They replaced superstition with knowledge and retained wisdom on their agendas for use of the new scientific knowledge. These two postures formed the basis of the new science.

For several centuries, scientists were heroes as they uncovered facts and enlarged knowledge of the material world, thereby facilitating an improved quality of life. But it is now becoming clear that more recently many scientists have jettisoned much of the ancient wisdom, values and community identity that enabled European civilisation to grow and flourish. Today our advanced Western civilisation is dominated by human activity devoted only to economic efficiency, much of it led by science combined with business. The superb levels of scientific knowledge revealed by rationalism and reductionism are often applied without wisdom and are challenging the foundations of the society that gave them birth. Climate change is a high profile example. Subjection of the food chain to science and global business is another area that threatens the future of humanity. That challenge comes not primarily from increasing world population, but from excessive consumption by the rich developed West combined with absence of wisdom in using knowledge and resources in farming and the food chain.

The wisdom, values and sense of community that brought Europe out of the Dark Ages into the present advanced civilisation have been rooted in Judeo-Christian traditions. The key positive life precept expressed succinctly by Jesus is to do good to others. Over the centuries, this principle has been the accepted standard that guided good judges, good lawyers, good governments, good business leaders, good educators — and good scientists.

Founders and Successors of Modern Science

Some scientists today assume that the worldview of scientists has always consisted only of knowledge. However, we have already seen that science was birthed by both knowledge and wisdom. The neglect of wisdom is of recent origin starting at the end of the 19th century and accelerating towards the end of the 20th century. This movement has been greatly influenced by genetics and the new concept of the selfish gene leading to a value system emphasising individual competitive success in which community does not matter. As we look back to the scientists on whose shoulders we stand and to whom we owe our privileged opportunities, we see that for centuries many of them were gentlemen-amateurs like Darwin and Mendel or they were individuals working in an academic environment supported by funding that did not press them for economically important results. The older universities were not founded to increase gross national product (GNP) but as centres of learning and teaching to improve the quality of life over the centuries. Many of the early and great scientists, if not the majority, saw their discoveries in the context of the whole of life. Their wish was to explore God's creation and to do good.

Some historians consider that modern science was born out of the Renaissance, Reformation and Enlightenment because the new scientists gave up superstition and believed the physical world had been made by the God of the Bible who is rational and consistent. They brought into science the values of European society that were based upon Judeo-Christian teaching enlightened by the Reformation. For them knowledge gained by science and wisdom derived from the transcendent view of life were part of the same world view. Doubtless some were cultural Christians, but many of the most famous scientists were active in their Christian life and stated that commitment in their writings. Each of these famous scientists were practising Christians: Galileo, Priestly, Newton, Pasteur, Lavoisier, Kepler, Faraday, Copernicus, Maxwell, Pauling, Planck, Fermi, Gauss, Dalton, Linneaus, Pascal, Boyle, Hertz, Marconi, Kelvin, Mendel, Dobzhansky. Exemplifying their world view Joseph Priestly, the 18th century scientist who discovered oxygen, wrote: "The contemplation of the works of God should give a sublimity to the scientist's virtue, expanding his benevolence, extinguishing everything mean, base and selfish in his nature".

If today we argue that the transcendent world view of these leading scientists was simply a part of the culture for their day and age, then we need to think seriously about how much our own scientific view reflects the materialism of the 21st century that makes no place for transcendent values. Unless we consciously consider our values and beliefs we inevitably become participants of the current dominant world view that directs resources and makes decisions only on the basis of economic and biological efficiency. Interestingly one of our fellow biological scientists, Francis Collins, who was for many years Director of the Human Genome Project, writes (Collins, 2006) that as a scientist he was a committed materialist until he realised in mature life that he had never looked at the evidence and data for transcendence and God. His examination of the data convinced him intellectually, partly through the writings of C.S. Lewis (1898–1965), that he must take the Creator into his world view.

Today, scientists face a new and deeper problem. Scientists used to be regarded as trustworthy. Not today. As a profession we are seen by many as fallible humans who have sold ourselves to business and who thereby have lost interest in serving society. This is an especially critical problem for scientists working in agriculture and food who earlier had been highly successful in averting the famine predicted by Malthus (1798). Today, scientists working in the food chain have lost their heroic status and even their credibility. The false assurances and scandals over food safety and negative effects arising from intensive farming systems have raised public suspicion. The Enlightenment opened the door not only to modern science and wisdom but also to capitalism and democracy. Sadly, science in the food chain has become a bed-fellow with elite capitalism while democracy and wisdom are ignored and sometimes abused.

The ancient values of goodness and concern for others maintained community that was itself the infrastructure for all activities. Goodness is now a neglected word, replaced by efficiency and profit. Western civilisation has degenerated into a singular focus on self interest. Few look after the values of community. The public square is largely devoid of goodness. Looking back we can see that modern Western society has used the dualism of Descartes not only to reject superstition but also to throw out values of community, belief and goodness. Today, materialism rules. We are paying the price.

THE PRESENT WORLD SITUATION

Although transcendent values have been sidelined while materialism, financial profit and consumerism dominate the scene, occasionally religious leaders have spoken about the terminal nature of this world view. The more sensible spokesmen among them have called for inclusion of transcendent values in the daily routines of life, to build positive human communities and to restrain human brutalities based only upon self-interest. Under economic prosperity, most people have turned a deaf ear to such views. Now an extraordinary and radical change has taken place among the prophets. Today secular leaders proclaim the end of civilisation as we know it unless we change. Many now take up the theme of the religious leaders in calling for ethical behaviour based upon transcendent values in the public place and in the market and for government regulations based upon these ancient human values. We frequently hear from secular leaders in all areas of life including a few top scientists, for example, astrophysicist Professor Martin Rees, President of the Royal Society and Master of Trinity College Cambridge, that Payback Time has already begun and it may be too late to change (Rees, 2003). We are on a course of self-destruction. The threats we create come from human actions motivated by greed and short-term benefits that erode sustainability in human institutions, in the environment and in the community of life. Most people agree intellectually but do little to change.

Thirty years ago, one of the earliest secular leaders to foresee this sorry state of affairs was Alexander Solzhenitsyn, who, speaking at the 1978 Harvard University Commencement Ceremony, predicted the end of Western civilisation. He had confronted the evil Soviet system and after suffering in the Gulag had been expelled from his own country and was living at the time in exile in the USA. His view was that our worldview of materialistic consumerism would fail. He noted that the West was already committed to humanism and had rejected the transcendent values of historic Christianity that had guided Europe and its cultural colonies for nearly two millennia. We now know he was right. Thoughtful leaders in the 21st century are asking for those transcendent values to be brought again into the fabric of life and commerce. Scientists have a special role in re-introducing ethics in the food chain which in recent decades has become simply another global business.

Molecular biology and genetic manipulation are currently the frontier topics of science especially in the food chain. How do they and the scientists working in this field fit into the present world view that has been described? Two characteristics are central. First, molecular biology is highly complex and knowledge of it is minimal. We are dipping our toes into an ocean of integrated systems that will take decades to measure and to understand. Second, this universe of molecular biology lies at the heart of life. Everything we change, or even try to change, in the plant and animal agro-bioresources of the planet carries implications for unknown effects on life in its many forms. We are at the frontier of what it means to be human and civilised. That is why we need to stop and carry out 'due process' in the scientific realm before seeking to modify the genomes of livestock. Our knowledge of the inner universe of the mammalian genomes is primitive. Accumulated human wisdom should warn us that tinkering with the genomes of other mammalian species so close to our own is fraught with danger. It is time to question the Enlightenment motto that "Man is the measure of all things". As scientists in the food chain, we can look back to a success story of increasingly cheap and surplus food in the West since 1945. That transformation involved substantial inputs from governments and societies in addition to science and market forces. Now we must realistically address the needs of the billions of poor and hungry at a higher moral level than simply viewing them as a market from which we can make money. We need to return to the couplet of doing good science and also doing good.

GENETICS AND REVISIONIST GENETICISTS

Where did Genetics come from?

Genetics is relatively new. Before 1850 heredity was an open field of speculation. The mechanics of heredity were not understood, but from the time of settled agriculture, about 10 000 BC, farmers started to domesticate plants and animals which they slowly improved by phenotypic selection over many millennia. Charles Darwin (1859) worked only with phenotypic observations and comparative biology but was able to conceptualise the process of natural selection without knowledge of the genome. Gregor Mendel (1866) broke new ground by using phenotypic measurements to realise that discrete units of genetic material pass from one generation to the next, but he did not know anything about their structure. From Darwin and Mendel knowledge of genetics grew though the 20th century into the Neo-Darwinian synthesis. But it was nearly 100 years after Darwin that Watson and Crick (1953) discovered the architecture of DNA revealing it as the common molecular language of all life forms separated by species boundaries. Crick hypothesised the existence of a 'messenger' molecule which carries the genetic information from

the gene and facilitates assembly of specific proteins. This assumption was affirmed by the discovery of messenger RNA (mRNA).

In 1958 Crick announced the 'Central Dogma of Molecular Biology' and later wrote of it in Nature (Crick, 1970): genetic information flows from DNA to RNA to proteins with the possibility of some flow from RNA to DNA. But the Dogma asserted that information never flows from proteins to nucleic acids. The generalised model was described as replication, transcription and translation with all the interest focussed upon DNA as the controlling molecule of the genome. The discovery of reverse transcription RNA in retroviruses like HIV was a challenge that was given inadequate attention at the time. Some scientists questioned the Central Dogma. But mainstream thinking extended the Crick model by linear thinking to incorporate the slogan "one gene = one protein". Hence, in the search for means to transfer genetic traits from one species to another, the gene has been the supreme target. This ability to move DNA artificially was developed in the 1970s and was first called recombinant DNA.

World-class scientists were so awed by this new technology that in 1974 they met and agreed in the Asilomar Moratorium to suspend further work on gene transfer until the process could be better understood. Today, safety standards in most research laboratories are maintained to prevent escape of GM micro-organisms. But global release of GM crops for food was launched in the 1990s and continues. Approval by national regulatory authorities is required for these releases. The primary government authority is the US Food and Drug Administration (FDA), which normally makes its decision using only data supplied by the corporation seeking approval for its GM product. This regulatory process has been criticised for its lack of independent verification and longer-term testing — an issue that is discussed later in the context of GM crops which, having been released, have failed to meet performance claims.

DNA molecules lie at the root of the integrated genomic and proteomic systems that define form and function in all living organisms from viruses to man. This awesome power of DNA derives from the way it is assembled, making it analogous to a language that can be marshalled into words, sentences, paragraphs, chapters and an infinite number of books. That analogy has been well made by Collins (2006) in "The Language of God". Thus, the 50 years since the structure of DNA was clarified have seen astonishing progress. However, recent research reveals that gene expression involves much more than DNA acting unilaterally. The genome is an integrated system with many levels of control.

Scientists now understand the basic structure of DNA and have the ability physically to invade inner molecular space and to manipulate the genome leading to changes in gene expression though not yet in predictable ways. But we lack adequate knowledge of how life functions at the molecular level. In our enthusiasm for using the limited knowledge, we construct models of molecular function and use them for genetic modification. But our models are inadequate and the process of making a functioning GMO results in many failures, details of which have rarely been published in the scientific literature and sometimes concealed. In New Zealand in 2009, for example, failure to publish details of abnormal animals resulting from transgenic research is being contested in the courts. Considering that the stability of the mammalian genome is the result of millions of years of trial and error with many discarded non-functioning individual organisms, these negative cases of genetic modification by human intervention are not surprising.

Revisionist Genetics in the 21st Century

The story of scientists is not always splendid. While the scientific method is objective and amoral, scientists are human and susceptible to all the failures and foibles of mankind. Scientists have sometimes advocated use of incomplete or wrong models, explanations and world views that later they have had to correct. That is part of the scientific process. Models are invaluable for research but need to be thoroughly tested and proven in controlled conditions before public and widespread projects are built upon them. Thus wisdom often calls for more knowledge before action. A tragic case of basing public policy on limited knowledge concerns mad cow disease or bovine spongiform encephalopathy (BSE). Top UK scientists later had to change their model and then retract their earlier assurances to the public that beef from affected cows was safe to eat and that mad cow disease does not affect humans. Many people have died. Only 50 years ago among cosmologists seeking to understand the origin of the universe, the idea of a Big Bang was a joke, far less popular than the hypothesis of Continuous Creation. Similarly over the last 100 years, genetic models to explain inner molecular space have changed. Blending has been discarded along with Lamarckism and Soviet genetics that emphasised the inheritance of acquired characteristics. Society has also had to contend with the abusive social constructs of eugenics and racism advocated by a few high profile scientists and backed by some politicians, social reformers and philanthropists. The realisation that DNA is the universal material of heredity tempted mainstream geneticists to presume that Crick's Dogma was the final model. But recently, in the early years of the 21st century, research has shown fundamental flaws in this model revealing a far more complex genome. Following the familiar path of science we must now engage in revisionist genetics for the 21st century.

It would be folly to release GM livestock into the poor areas of the developing world where livestock is one of the key resources of the people. Our models are inadequate and we know so little. It is like launching a rocket to take people to the moon before the co-ordinates of its motion have been comprehensively measured and understood.

Crick's contribution on the structure of DNA and his prediction of mRNA were brilliant, but his Central Dogma was misleading and became the working model for many scientists until recently. Forty years after Crick announced his Central Dogma a major shock came from the Human Genome Project (HGP). The one gene = one protein model and the sheer quantity of human DNA had fostered an expectation that the hundreds of thousands of known mammalian proteins were associated with up to half a million human genes. In fact the HGP found only about 23 000 genes, little more than the nematode *Caenorhabditis elegans*!! The discovery of the low number of human genes added credibility for a time to the earlier concept: 'Junk DNA'. That model claimed to explain the large quantity of human DNA by postulating that much non-coding DNA was an inert residue from evolutionary development – useful at one time but now junk. However, high profile researchers in different centres now assert that since coding genes represent only about five percent of DNA it was a wild statement to assert the rest was junk. We now know that at least 50% of DNA has specific functions though most of the functions are still not documented. Transposons and jumping genes, whose roles are often unclear, also occur frequently. We do know that much DNA, formerly called junk, plays a key part in gene expression. Mattick (2005), from the Institute of Molecular Bioscience, University of Queensland, challenges the orthodoxy that 95% of DNA is evolutionary 'junk' as follows: "Most of this DNA is transcribed into non-coding RNA and consists of a hidden layer of

gene regulation that directs the development of complex organisms. Expression depends on which tissue the genome is directing".

So in the early years of the 21st century, three assumed models — the Central Dogma, one gene = one protein, and Junk DNA — have all been buried. Today our understanding of mammalian genes has changed. We now see that:

• genes multitask;
• genes are interdependent;
• genes overlap in function;
• information flows both to and from genes;
• switches can modify gene expression;
• the genome is highly integrated, compact and efficient.

Some of these characteristics of genes are explained by alternative splicing. In forming mRNA and proteins, some genes contribute information from only part of their DNA which is joined to limited sections of DNA from other genes, thus opening the door to numbers of proteins disproportionately greater than the number of genes. All this new information is significant for genetic modification of livestock because it will be difficult to anticipate all the effects of transgenes.

Analysis of the Human Genome – ENCODE Project

A further important contribution to the revision of genetics has been published. The ENCODE (2007) project involved 80 scientific teams in 11 countries over five years and cost $42 million. This mammoth study was a further step beyond the HGP analysis of base pairs. The ENCODE project analysed 1% of the Human Genome in detail and concluded, *inter alia*, that: "the genome is pervasively transcribed, such that the majority of its bases can be found in primary transcripts, including non-protein-coding transcripts, and those that extensively overlap one another". Further, the ENCODE scientists say that: "integration of the new sources of information, in particular with respect to mammalian evolution based on inter- and intra-species sequence comparisons, has yielded new mechanistic and evolutionary insights concerning the functional landscape of the human genome". They conclude that RNA has astonishing tasks, even controlling genes. The mammalian genome can no longer be viewed simply in terms of autonomous genes, since it consists of a complex integrated community of molecules. For example mRNA not only comes from protein coding genes but also from many other parts of DNA whose function is, as yet, unknown (Check, 2007). These coding sequences appear to be widely scattered throughout the genome (Callinan and Batzer, 2006), probably as protection against transposons and retroviruses being randomly inserted with disruptive effects upon the code. Greally (2007) in an Editorial in Nature on the ENCODE Project argues that we must go back to the beginning of molecular genetics. The Editorial reaffirms the point made by Mattick (2005) that certain regulatory processes are specific to cell type and further research into the functioning of the human genome will have difficult choices to make on which cell types to study. The realisation that gene expression is dependent upon cell type, as described by Mattick and the ENCODE project, effectively places a time bomb under the assumption that genetic modification of livestock by transgenes and cloning will be easy, predictable and safe.

The *Bos taurus* bovine genome was recently analysed for the first time (Elsik et al., 2009) and was found to have a minimum of 22 000 genes, not much different from the human genome. The study facilitates comparison between the two genomes to identify highly conserved DNA, specific break points in the evolution of cattle, frequency of repetitive elements, etc. However exciting these comparisons may

be for research, the similarities are also warning signals against premature release of modified bovine genomes into food production, especially in developing countries where livestock are herded on wild herbage and their milk and meat is consumed by humans. The BSE saga speaks into this situation especially as genes involved in metabolism are highly conserved.

Other current areas of genetic research contribute to the call for caution and more knowledge as they identify further factors likely to complicate the way in which the mammalian genome functions; for example, stress proteins that act as chaperones to the DNA against heat and other environmental stress (Calderwood, 2007) and endocrine disrupters that affect gene expression (Kortenkamp, 2003). Another major factor is the realisation that epigenetic effects are more common than earlier thought. They include not only methylation, a well-known and common cause of modifying gene expression, but also discoveries of flows of information from other sources that arise in widely separated areas of the genome. For example, feedback information has been discovered from cell tissue to RNA to DNA that affects the way a gene operates. The detection of many forms of RNA with differing abilities to affect gene expression, described by the Nobel Laureate Cech (2004), is a revolutionary finding when viewed against the former model of one gene = one protein; earlier models must now be regarded as simplistic. Further challenges to understanding the genome are emerging from clues that information flows from the external environment to cell tissue and thence to possible modification of DNA expression. This possibility raises important issues concerning livestock adaptation that would be relevant if GM livestock were placed in the tropics.

MOLECULAR BIOLOGY, LIVESTOCK AND THE POOR

Mixing Advanced Science and Subsistence Farming

What do genetic manipulation of livestock and the poor have in common? They belong to completely separate worlds. Molecular genetics is at the cutting edge of Western science where it is buttressed by the resources, facilities and infrastructures of the most advanced civilisation in the history of the world. Its practitioners are highly educated scientists working in controlled laboratory conditions with access to finance and equipment. They are beneficiaries of a high standard of living and quality of life in an urban society where the system works, food is plentiful and cheap, life expectancy is high, employment opportunities good, government stable and the vagaries of the natural environment and the weather are largely irrelevant.

There are about three billion poor in Africa, Asia and Latin America whose lives are totally different. They live in simple conditions on $2 a day or less often in remote locations where they are exposed to the uncertainties of the natural world with little opportunity for employment, health care, formal education or prospects of change. Their worldview is more similar to that of pre-Enlightenment Europe than to that of the West today. Land and livestock, including poultry, are their major resources for security of life and food. Those who do not own land or animals are nevertheless dependent upon a rural community where these resources support the local economy. Livestock are wealth and insurance against an unknown future where government assistance is absent or very limited.

How can these two worlds be brought together? Should the attempt be made to unite them? One is strong in science and the other strong in community values and practical knowledge of how to survive in a hostile environment. Have the poor been asked if and how they want their livestock changed? It would be very difficult for the West to ensure Prior Informed Consent because of the paucity of knowledge of the genomes of GM livestock and the unknown

consequences. Ethics should be a major issue in the genetic modification of the livestock of the poor.

Consumers in Europe made strong statements against the GM crops when they first appeared in 1999 and labelling is now mandatory in the EU for the few GM foods allowed. More recently, some EU governments have stated their opposition to GM crops being planted experimentally. The EU parliament debated cloned livestock for food production in September 2008 and the vote was overwhelmingly against milk and meat products from cloned animals entering the food chain — 622 against, 32 for with 25 abstentions. It is rare to have such a strong vote on any issue. Should the view of European consumers affect the issue of GM livestock being introduced to the developing world? There is a frightening gap between the negative view of the European population and the posture of many European scientists who are confident that these new molecular techniques will bring increases in milk and meat production. Research directed to this end is in progress in both private and public institutions with the aim of using transgenic livestock in the food chain as soon as possible.

Proponents of transgenic livestock have serenely announced already that the technique will produce animals with superior qualities (Biotechnology Industry Organization, 2008). This organisation represents the companies and their scientists working in this field and speaks of enhancements to almost all aspects of livestock production: milk and growth, carcase composition, animal health and welfare, nutrition and public health, environment, hair and fibre - all are listed heralding a new era in animal science. These changes are described as though they are guaranteed rather than simply potentials visualised by scientific visionaries and speculative business interests.

Because of the difficulty of producing a GM animal, one plan is to use somatic clone nuclear technology (SCNT) to multiply superior GM individuals as a means of spreading the transgenes throughout the commercial livestock population using enhanced reproductive techniques such as artificial insemination and embryo transfer. This strategy for transgenic livestock opens a vast minefield of uncertainty that would need extensive and time-consuming research over several generations. The effects of producing successive generations of GM livestock by techniques that by-pass the filtering effects of gene segregation must be researched before commercial populations are committed to this new policy. Some surmise that gametogenesis will be an effective filter against abnormalities. We simply do not know. Such ignorance is dangerous. Some scientists argue that these dangers are greatly exaggerated because GM animals simply have their DNA coded differently and people have been eating DNA since the beginning of time. The crucial point is that risks emerge not from eating DNA *per se*, but from the harmful proteins that transgenic DNA may produce in a recipient organism — and these are unknown. Thus, the hazards are more likely to appear in the proteomics not the genomics. Most research is directed to the latter and not to proteomics which is a very new discipline.

The approval by the US Food and Drug Administration in 2006 of clones of cattle, pigs and goats for production of food products opens the way for SCNT clones to be used to propagate GM livestock. The complexities of using these technologies in commercial livestock and the human food chain raise a variety of concerns that have not yet been adequately researched. For example, genes from animals produced by SCNT have already taken part in cell differentiation during which specific genes are switched on and off. The importance of cell type in gene expression raises deep questions since an SCNT genome derives from an arbitrary choice of a somatic cell type that is then triggered artificially to start again as an embryo. The activity within a cell involving genes, DNA, RNA

and proteins is affected by cell type. This fact raises a fundamentally important question of which somatic cell type and therefore which specific complex of molecules should be chosen to form the genetic profile of the SCNT clone and of the ensuing livestock population. This substantial question has not been tackled by planned experiment, but has been partially addressed by default as large numbers of failures occur before a live and apparently healthy SCNT animal is achieved. Dolly resulted from the 276th attempt. Mattick (2005) and the ENCODE (2007) project anticipated the problem in general without reference to SCNT when commenting upon the huge research task that lies ahead — to examine the expression of the genome of each cell-type. Use with livestock at this stage is risky. The unknown hazards of the type mentioned have been recognised as a possibility by the US Department of Agriculture which has taken the realistic step of wanting to compile a record of cloned livestock on farms - no doubt to facilitate tracing in the event of problems arising.

The Scientific Imperative or Empowerment of the Poor

Scientists working in this field appear to operate under a scientific imperative. A novel technique has been discovered enabling scientists to manipulate the genomes of livestock species. The methods are far from perfect, animals suffer in the process and knowledge of the molecular micro-universe is scanty. European citizens have expressed their wish to keep their food chain free from such products. Nevertheless scientists speak confidently about increasing the world food supply of meat and milk with these techniques. In their thinking the research stage has been passed. The moral high ground is invoked by claims that this new technology will help feed the world and is the legitimate scientific successor to the Green Revolution. Much more research is needed leading to comprehensive independent evaluation followed by consultation among the many stakeholders. Decision-making is then rightly in the hands of those who will benefit and carry any risks. A broader point currently ignored by the scientific imperative is the possibility that GM livestock will turn important segments of the consuming public against meat and milk. There seems little doubt that the growing demand for organic products in the more affluent food markets has been helped by the appearance of GM crops. The perception of spoiling your own market for animal products requires wisdom which, surprisingly, seems absent from the scientific imperative.

Assessment of Risk

The field of GM livestock faces Black Swan Events — defined as "unknowns that have a large impact but which are hard to predict". Mad Cow Disease (BSE) was a Black Swan event in the production of food from livestock. The defence against Black Swan events is not to be found in conventional use of probability statistics which are commonly used in biology. Even one Black Swan is one too many. The defence against such events is wisdom. This means looking at the scenario from every possible angle and then making a prudent decision based upon the likely scale and reversibility of any negative consequences if such were to appear.

Lessons for GM Livestock from the GM Crop Saga

Livestock scientists can benefit by the lessons arising from GM food products that have been on the market in the USA without labelling since 1998. Although accepted in the USA, they were initially rejected by consumers in the EU and approved in 2004 only with labelling. A widespread public controversy still over-shadows GM

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crops with opposition from environmentalists, from farmers serving the growing organic market whose crops are sometimes polluted by GM crops growing nearby, from consumers who fear for their health, and from some independent scientists. An independent assessment of the first ten years of GM crops from 1998–2008 is now available (International Assessment of Agricultural Knowledge, Science and Technology, IAAKSTD, 2009), This study was made by 400 researchers worldwide working under the independent umbrella of the World Bank and multiple UN Agencies and was led by Dr. Robert Watson who is a high profile and experienced international scientist. The authors exhaustively examined the peer-reviewed publications that could contribute to an assessment of science and technology used in agricultural production including both developed and developing regions. The report concludes that GM crops have not, on average, increased crop yields. In some cases output was transiently increased and in others it was reduced, but this global study found no sustained increase in yield levels from GM crops. Farmers using GM seeds of corn, canola and soya are mainly large-scale even in developing countries such as Argentina, Brazil and Mexico. They use GM seeds to reduce their costs of spraying as most GM crops have transgenes resistant to more concentrated chemicals. The study found that reduction in costs and transient increases in production depend upon local conditions and crop management. The IAAKSTD study shows that GM crops to date cannot be regarded as a 'silver bullet' whose use will automatically increase food production.

GM crops were introduced to the market ten years ago with the promise of increased food production but without either adequate research or public debate. As a consequence a promising new technology has received a negative public image. The new science has been used almost entirely upon the four staple crops which are the most profitable markets in large-scale farming. The technology was promoted with the claim to feed the world better by producing crops that are able to grow in harsh and unfavourable locations. This has not been achieved to date and some critics see this claim largely as propaganda to promote the image of the four staple GM crops. The story revealed by the IAAKSTD study does not enhance the image of scientists involved with GM crops whether they work in the laboratory or as scientists advising governments on regulations and approval.

Consequences and Regulation of GM Crops

GM cotton (Bt cotton) is a non-food crop which Monsanto promoted among both large scale cotton producers and small scale peasant farmers especially in India. Proponents have considered it a GM success story while there have been anecdotal accounts of negative results and consequences. High levels of suicide among small scale Indian farmers using GM cotton have been recorded that allegedly were especially associated with credit taken to buy GM seeds and the subsequent inability to use harvested GM seed. The International Food Policy Research Institute examined this situation (IFPRI, 2008) and concluded that the documented increase in suicides could not be linked directly with the use of GM cotton and was due to institutional, climatic and economic constraints among many factors. Some farmers' associations in India have complained of livestock deaths after ruminants have foraged on the GM cotton vegetation in the fields following harvest. The Indian government extension services have undertaken to carry out controlled experiments on this issue. Monsanto has now disclosed that the cotton pest, pink bollworm, has developed resistance to Bt cotton in several regions of India (Monsanto, 2010). This response of naturally occurring organisms to GM crops was one of the dangers predicted by some scientists who, from the early days of GM crops, considered that it was

premature and dangerous to release transgenes in domestic crops into farming, the environment and the food chain. Understandably, large segments of the public have an image of scientists who have sacrificed objectivity and normal scientific protocols of transparency, failed to publish negative research results, and should have sought wider public debate before the products of the new technology were used extensively. One such scientist is Schubert (2002) a cell biologist at the Salk Institute and earlier a colleague of Francis Crick. It is difficult to avoid the conclusion that some scientists have become self-serving and that consequently society as a whole has not benefited from the new knowledge and technology.

A further important issue for scientists is the need to remain in touch with farmers, consumers and citizens on issues of GM food. If scientists become isolated from common people they are in danger of making decisions on the basis of the latest technology results and of pressures that commercial corporations place on governments for swift regulatory approval. For example, the Indian government in 2009 decided to give approval to GM Bt brinjal (aubergine) which is a widely used indigenous food source in India. Farmers and people from all walks of life protested and called a fast. Several leading Indian scientists, including Dr. M. S. Swaminathan of international stature as a plant breeder, spoke against the release of GM brinjal. The government changed its decision and GM brinjal will not be released. Dr. Swaminathan (2010) took the opportunity to repeat his view that GM crops should be subject to testing in an internationally qualified and independent laboratory as privately generated data are inadequate; further he considers there is a risk that untested Bt brinjal may be like tobacco and lead to chronic dosage problems; he also thinks that introducing GM brinjal will destroy indigenous varieties. Another highly placed scientist in India, Dr. P. Bhargava (2010) who serves on the national GM regulatory body has called for a freeze on GM crops on biosafety and health grounds before release.

The IAAKSTD study and the recent experiences with Bt cotton and Bt brinjal show how vital it is for scientists to combine wisdom with the new-found knowledge in molecular biology. These mistakes must not be repeated with livestock. The risks that may arise are too great for GM livestock to be put into the public domain in the developing world. The hazards include the failure rate such as abortions and birth of abnormal animals which could be catastrophic in the small herds and flocks of poor livestock keepers.

Lessons for GM Livestock from the BSE & vCJD Saga

The aims of those advocating transgenic livestock include substantial modifications to the life processes. Such changes clearly target key proteins — a scenario that opens the door to dangers from the unknown and unexpected proteins that will be produced and eaten by humans in milk and meat from transgenic livestock. The plan is to use gene transfer on livestock to change growth and lactation physiology, metabolism, endocrine systems and reproductive hormones of livestock. To be safe, such radical interventions will require decades of research to ensure that no unwanted proteins are produced somewhere in the mammalian body.

Transgenes are chosen because they are known to produce a desired trait in the donor species, but in the recipient species there is no guarantee that they are located correctly or supported by the appropriate RNA and protein components, enabling them to function as predicted in each cell type. Like natural mutants, transgenes are not inserted precisely and therefore experience a similar high rejection rate, already confirmed by experience. In the case of livestock the high failure rate highlights animal welfare issues. Many attempts are needed to produce one designer organism, indicating that most (alien mutant) transgenes are tested and rejected by

recipient species that have been on the evolutionary test bench for millions of years and have enduring homeostasis. The question must also be asked about generational stability in transgenic organisms. The process of meiosis is a powerful filter that any mutant must survive. Reversion to the wild type would undermine claims for increased food production and negate the purpose of the whole enterprise. By any standards of logic, the rushed ambition to sell products from transgenic livestock is courting a large harvest of abnormal animals and the possibility of economic failure for herd owners or misfortune among consumers before untarnished success is achieved. There is also a deep question of how a GM animal should be tested for normality before release for use.

The lesson of BSE (mad cow disease) and its human variant Creutzfeldt Jacob Disease (vCJD) speaks into the issue of transgenic livestock. These conditions are caused, so far as is known, by an aberrant protein (prion). The economic losses and increased costs were enormous; hundreds of thousands of cattle were killed; and 177 people died by 2009 from vCJD. The genetic aspects of this scientific study have been given too little serious attention in the plans to produce GM livestock. BSE was spread from cow to cow though eating the prion present in offal used as a feed supplement. People were affected by vCJD through eating the prion in beef from cattle with the condition. But not everyone who ate the prion died. Most of those who died from vCJD carried a specific allele combination at one codon in their genome. Other people with a different allele did not get the condition even though eating the prion. This fact points strongly to a genetic linkage between the bovine and human genomes. In view of the very close matching of human and bovine DNA arising from evolutionary history, this finding is not surprising. But it is a serious indicator of other unfortunate and as yet unknown linkages that may appear when humans eat meat from transgenic cattle. The lethal agent is not the DNA but the protein. The unknown BSE-type dangers that may arise from GM livestock are unlikely to be caused by animals or people eating transgene DNA. The risks are associated with the expressions of the transgene as unexpected proteins, either alone or more likely in combination with existing genes in the recipient organism with which the transgene interacts. A further complication is the possible effect upon wild mammalian predators that may eat GM livestock. The scenario of unforeseen transfers of harmful proteins from GM livestock to humans, to other livestock and to wildlife opens the door to Black Swan events too serious to be ignored. Everything points to the need for long and intensive research over many years and generations before GM livestock are released and used for food.

GM Livestock, Adaptation and Empowering the Poor

The idea of inserting genes into livestock by radical and untried methods for use in the developing world raises a host of questions about adaptation to local environments, feed resources, animal health, disease and parasite resistance. Any genetic modification has to focus upon a limited group of traits like growth, milk, etc. The effect of such genetic modification on adaptive traits will be a huge research project, for genotype-environmental interactions are well known to be difficult to measure and to interpret. Adaptive traits suited to local conditions that have been developed by natural selection over long time periods are key to the value of livestock to billions of poor in the developing world. Any deliberate or unintended modification in adaptation could have dire consequences for the herd owners. The IAAKSTD (2009) report addresses the issue of farming among poor small scale farmers in the developing world and comments that genetic modification of agro-bioresources is a feature of Western intensive farming that requires the support of infrastructures and capital that are absent in many rural areas of Africa, Asia and Latin America. In general the authors concluded that intensification of farming by technology transfer from the West has very limited value among the poor. This is a stark conclusion from an authoritative and independent body of researchers. The Assessment was also critical of the environmental impact of intensified Western agriculture and found it unsustainable. After the IAAKSTD Report, a pan-African study was published by UNEP-UNCTAD (2008) showing that agro-ecological agriculture, when properly applied in Africa, out-produced other technologies. The IAAKSTD Report advocates that research should be re-directed towards improving locally sustainable and high yield methods instead of technology transfer.

CONCLUSIONS

The molecular structure of life is complex and highly organised. It has been shaped by billions of years of mutation, testing, rejection and adaptation: an extraordinary process with remarkable results. A key feature is the astonishing way in which information is stored, accessed, transmitted and used for life processes by a remarkable integration of DNA, RNA, proteins and other molecules that is also open to information causing epigenetic effects. The enthusiasm for genetic modification of livestock needs to be tempered by the evident current lack of understanding of this highly integrated process. These facts merit serious reflection as scientists of this generation attempt to improve upon the natural product.

Enormous periods of evolutionary time were essential for producing the integrated species genomes that show enduring stability, economy of structure and efficiency of function. This process does not fit the pulse of science and business in the 21st century. Wisdom would suggest that the dash for swift returns on investment is highly unlikely to yield livestock genomes that are durable, functional and safe.

Scandals like BSE and its human form (vCJD), toxins in animal products, unlabelled GM food, foot-and-mouth disease epidemics, avian and, so-called, swine flu, have diminished the reputation of animal scientists in the eyes of the public, many of whom now see scientists as agents of big business. The historic image of objectivity and service to the public good has been reduced. Animal scientists must now, more than ever, take seriously their obligation to balance scientific knowledge with social wisdom in evaluating molecular technology in livestock production — especially in the rural areas of Africa, Asia and Latin America where populations are more vulnerable to change.

Citizens in Europe feel precarious and at risk from the activities of a combination of elite scientists and business interests whose self-appointed mandate to manipulate food has not been put through the democratic process and, in the USA, due to the government decision no labelling is required. Absence of labelling to identify GM products contravenes the principles of the market economy. The molecular biologist today is well equipped as a technologist with specialist knowledge. But the extreme reductionism of scientific education, research and practice combined with the priority given to mission-oriented research leaves many scientists deficient in two areas:

- absence of the grand vision of science held by many great scientists of earlier generations;
- lack of wisdom and understanding of the deep complexity of human life.

These two deficiencies are particularly relevant to the issue of using transgene technology and products to change livestock.

SUMMARY

Gene transfer for livestock may be evaluated in terms of Foundations, Fallacies and Assumptions.

Foundations

Knowledge has lost the underpinning of wisdom that is essential if science is to contribute to improved quality of life for society as a whole.

Fallacies

Some biological scientists have embraced an erroneous belief they can quickly produce 'better' species than evolution and millennia of domestication and selection.

Assumptions

The expectation that livestock owners want transgenic livestock and that consumers will accept milk and meat products from them is unethical and probably wrong.

Proposal for a Moratorium on the Use of GM Livestock for Food.

The author asks fellow scientists in the field of animal science and molecular genetics to consider making an independent statement of their commitment to the well-being of society at large. They should consider following the initiative of their predecessors who agreed the Asilomar Moratorium when they realised they had a new technology with great potential for good or bad consequences. Scientists should call for a Global Moratorium banning the use of genetically manipulated livestock for food. Research should continue until knowledge is sufficiently advanced to preclude negative effects when consumers can indicate in a referendum if they want milk and meat from GM livestock.

Postscript

Some great thinkers and people of action in the past have recognised that scientists are easily tempted by power through specialist knowledge.
- Michael Polyani: There is a danger of scientists coercing society because of specialist knowledge. They must not be allowed to seize levers of power and must work through democracy.
- Winston Churchill: Scientists should be on tap and not on top.
- Albert Einstein, Bertrand Russell, Linus Pauling, Andrej Sarhkarov: All warned of the dangers of scientific anarchy.

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Merino Breeding Program Improves Wool Production in Western US Range Sheep Flocks

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ABSTRACT

A Merino breeding resource flock was established at Rafter 7 Ranch, Yerington, Nevada. Initially, 500 Rambouillet ewes were purchased from two established breeders in 1990. These ewes were bred naturally or by artificial insemination (AI) to imported Merino rams from Australia and to crossbred rams selected within the flock. The flocks were expanded to 1300 ewes and bred in 30 single-sire mating groups as of the 2006 breeding season. Flock management is in two breeding lines, one as a registered Rafter 7 Merino flock (n = 650) and the other (Merino × Rambouillet) as Rafter 7 Line (n = 650). The spring lambing flock winters on desert rangelands, is grazed on irrigated pasture from shearing through lambing and early weaning. Compared with the original base ewe flock, Merino and Merino crossbred ewes produced higher clean wool yields, longer staple lengths, and higher grease fleece weights. The body weight and greasy fleece weight showed a significant ($P < 0.05$) difference between two flocks whereas no differences were observed for wool fibre diameter, length and comfort factor in most recent analysis. However, fibre diameter variation was significantly different ($P < 0.05$) between the two flocks for age groups and birth years. Body weight, fleece weight and fibre diameter showed significant ($P < 0.05$) but low to moderate correlations. Approximately 1000 breeding rams and 500 replacement ewes were distributed to commercial range flocks in the western states. The dissemination of introduced Merino genetics in the western range sheep flocks is expected to enhance wool quality and wool profits in the western region of the USA.

Key words: *Merino, breeding, genetics, wool, fibre diameter, rangelands.*

INTRODUCTION

In the United States, sheep and lambs are raised primarily in small farm flocks in the Midwest and the East, and on large ranching operations in the West (NRC, 2008; www.nap.edu). The first domesticated sheep were brought to the United States in 1493 with the second voyage of Columbus. With growing importation of Spanish Merino sheep in the late 1700s and early 1800s, the U.S. wool clip began to grow substantially. As the industry moved to western states, wool production from the French Rambouillet, originally developed from Spanish Merino genetics, expanded, and by 1870, about 80% of all USA sheep were of Merino origin (ASI, 2002).

However, the USA sheep inventories declined steady from 56 million head at peak of 1942 to the present day seven million head. Nevertheless, sheep grazing in the western rangelands can be profitable and environmentally sustainable (Glimp and Swanson, 1994). Most of the sheep inventory of the country is in wool-meat dual-purpose sheep and the majority of flocks produce medium to strong wool of 23.5–26.4 micron (spinning count in 58–60s). In 2006, the 14 western range and intermountain states accounted for 72% sheep and lamb inventories but produced 77% of the USA wool clip and received 88% of income from wool sales (USDA, 2007). The USA clean wool sale price analysis indicated that every one micron decrease in fibre diameter of fleece (for example 22 micron to 21 micron) increased by 10% the market value of USA produced wools (Anderson et al., 2007). Over the last two decades, the fine wool clip has become progressively finer while textile technology has improved for processing superfine wool types. The consumer demand of a light weight, next to skin comfort wear, and fashion trends drive the world apparel wool markets. Consequently, the international raw wool trade premium prices set for fine (19–21 micron) and superfine wool (16–19 micron) categories have increased significantly over the other type of wools during 1990s (Land, 1990; Purvis, 1995; Wuliji et al., 1999; Wuliji et al., 2001), which facilitated the Merino breeding projects in some wool growing regions. While the size of the USA sheep industry is expected to be stable with possible slow growth in future, in order to be profitable, sheep producers should take advantage of both domestic and international fine wool niche markets, and the biological ability of the sheep to control weeds and thrive in suboptimal ecosystems (Lupton, 2008). This paper describes a wool sheep selection programme at the Rafter 7 Ranch and the impacts of Merino genetics dissemination into western USA range sheep flocks. Animal performance and wool characteristics were analysed and are presented for two selection flocks, namely, Rafter 7 Merino and Rafter 7 Line.

MATERIALS AND METHODS

Flock Establishment

Two decades ago, the University of Nevada-Reno and Rafter 7 Ranch established a Merino breeding programme at the Rafter 7 Ranch near Yerington, Nevada to introduce superior fine-wool Merino genetics from Australia to provide genetically improved and adapted breeding rams and ewes for the U.S. western range regions. The sheep flock at the Rafter 7 Ranch has been managed as a quarantine flock since its establishment. The only live animal introductions to the flock have included seven Merino rams imported from Australia in 1990 and ten Merino rams imported in 1997, which were quarantined and tested in Australia for 90 d and further quarantined and tested in the USA for 30 d to meet USDA–APHIS importation requirements. Frozen

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semen, collected from quarantine rams in Australia to meet USDA–APHIS requirements, has been imported from another 32 rams. The flock met the USDA–APHIS Certified Scrapie Free certification requirements by 2005. The average fibre diameter (AFD) of the original imported rams was in range of 17.5–19.5 microns according to the stud book information, while mixed age Rambouillet ewe flock fleece were estimated at 58–60s spinning counts (objective test was not available at the time) during the initial crossbreeding phase. All animals born on the ranch are provided an individual metal ear tag at birth and an electronic ear tag number incorporated into their scrapie tag at weaning at approximately 90–120 d of age. Sheep numbers have steadily increased to meet the needs for breeding, replacements, and distribution of breeding stock to sheep producers. The flock is physically inventoried at breeding (approx. Nov. 20), shearing (approx. March 20) and lamb weaning and ewe culling (approx. Aug. 15), and inventory changes due to sales and animal death losses are recorded.

Range and Pasture

A flock of 1 300 breeding ewes and 35 stud rams are maintained at the Rafter 7 Ranch, a University of Nevada-Reno (UNR) cooperative sheep station owned by the Edwin L. Wiegand Trust. The ranch includes 1 400 ha of private land and grazing permits on 40 500 ha of Bureau of Land Management Lands, and 1800 ha of USDA forest land. The flat pasture elevation is at 1200 to 1 500 m, and high desert range elevation is up to 3000 m. The annual precipitation within the area of perimeter is less than 200 mm, mostly as winter snowfall with unpredictable frosts and wind patterns. Desert shrubs include black greasewood, basin big sagebrush, black sagebrush, bud-sage, white sage, and ephedra. Grass species include Indian ricegrass, bottlebrush squirreltail, and cheatgrass. The established pastures were primarily tall fescue, over-seeded with Ladino clover. Improved irrigated pastures include a mix of tetraploid perennial ryegrass, improved fescue cultivars, a grazing variety of alfalfa and Ladino clover. An additional 50 ha of irrigated land is used for alfalfa hay production and aftermath grazing. Irrigated pastures, 35 pastures at 2–6 hectares, are set stocked during breeding and lambing, with an intensive rotation the rest of the grazing season.

Animal Breeding and Distribution

Natural mating and AI were used alternatively during the upgrading phases. A computerised record and data base program that includes individual animal pedigree, sex, birth date, birth and rearing rank, weaning and yearling performance record file is maintained on the ranch. Two seasonal lambing managerial options were adopted since 2006 although the majority of lambs are scheduled to be born during spring lambing. Animal selection was made each year prior to the breeding season on a multi-trait Performance Index in conjunction with 'independent culling' for undesirable traits such as poor conformation and structure, wool face cover, jaws, infertility, and coloured fibres. Animal selection was based on objective wool measurements as well as subjective assessment, growth rate, and reproductive performance traits.

There are five wool breed societies established in the USA including American Cormo, Boroola Merino, Rambouillet, Debouillet, and Delaine Merino (ASI, www.sheepusa.org). The Rafter 7 Ranch Merino flock was fully inspected, pedigreed and registered with the Delaine Merino Breed Registry. Flock management is in two breeding lines, one as a registered Rafter 7 Merino flock (n = 650) and the other (Merino × Rambouillet) as Rafter 7 Merino Line (n = 650), both of which are selected for high fleece weight and quality, twinning,

and growth traits. The spring lambing flock was wintered on desert rangelands, and grazed on irrigated pasture from shearing through lambing and early weaning. Lambs were subjected to pre-selection culling at weaning and final selection based on yearling performance including body and fleece weight, and wool characteristics. A final selection performance index was derived by various adjustment weightings to birth and rearing ranks, age, body weight, weight gain, fleece weight, fibre diameter and length. Ram distribution catalogues for selected rams and ewes with a comparative Performance Index were posted to the potential sheep producers/clients 2–4 weeks before the ram sale field day on the ranch. Selected rams and ewes were presented with IDs, pedigree and yearling performance data sheet, and health certificate in subdivided pens on the field day. Regularly, about 70–100 producers participated in an annual ram sale and several dozen ranchers purchased their choices of breeding rams and ewes by an open bidding at the field day auction. Genetic distribution and impacts on range wool sheep production were monitored on a number of ranches who consistently used Rafter 7 Ranch rams. Four of these associated ranches located in Reno, Ely, Fernley and Rafter 7 of Nevada were surveyed for their superfine category (<19 micron) wool lot weight ratios in the clips using the wool warehouse records and public auction information from 2004 to 2009 wool sale catalogues.

Wool Production and Clip Preparation

Individual fleece weight and wool characteristics were recorded for the life time of breeding ewes and rams. Pre-shearing midside wool staples were collected from each sheep and a set of programmed wool tests for wool characteristics, including average fibre diameter (AFD), fibre diameter variation coefficient (FDcv), average staple fibre length (ASL), and estimated comfort factor (CF) were measured using an OFDA 2000 instrument (IWG Pty Ltd, Australia). Shearing was scheduled at least four weeks prior to lambing. Fleeces were classed according to the pre-shear test classification with some subjective alternatives, such as short, discoloration or tender strength. Wool clip volumes and sale values were recorded and presented for last five years. At the 2009 spring shearing, approximately 3000 fleeces at Rafter 7 Ranch were pre-tested, shorn and classed into five category lots, namely, ultrafine, superfine, fine, medium and coarse lines, which were baled and transported to the wool warehouse that provided a sale lot test certification.

Measurements and Statistics

Animals were recorded for selection flocks, post-shearing body weight (BWt), greasy fleece weight (GFWt) and wool characteristics including AFD, FDcv, CF and ASL using the OFDA 2000 instrument on pre-shearing midside staple samples. An annual wool fibre diameter measurements of 556 selected mixed ewes in two flocks, which were born over four birth years in 2001–2004, were monitored for five years of wool production (five shearings) respectively. Therefore, the changes in AFD were compared to every year from the first to the fifth shearing (Age I, II, III, IV and V) consecutively. Post-shearing BWt, GFWt and wool characteristics of two flocks (2009, observed n = 2 218) were analysed. The procedure of GLM, CORR, and GLIMIX of SAS (SAS Inst. Inc., Cary, NC) were followed for data analysis of BWt, GFWt and fibre characteristics, and fibre diameter variations in birth year and ages.

Table 1. Means of body weight (BWt), fleece weight (GFWt), fibre diameter (AFD) and fibre diameter variation (FDcv), staple length (ASL) and comfort factor (CF) in flocks (2009 shearing).

	No Obs.	BWt (kg)	GFWt (kg)	AFD (µm)	FDcv (%)	ASL (mm)	CF (%)
R7 Merino	1 291	66.5^b	5.32^a	19.4	17.2	86	99.0
R7 Line	1 947	72.2^a	4.63^b	19.5	17.4	82	98.9
SE		0.95	0.9	0.1	0.05	0.5	0.05

$^{a,\ b}$ Column means with different superscript letters are different (P < 0.05).

Table 2. Least squares means of average fibre diameter of Rafter 7 Ranch breeding ewes by flock and age group (n = 556).

Flock	Means /Flock	Means by Age Group					SE
		Age I	Age II	Age III	Age IV	Age V	
R7 Merino	20.5	18.4^e	20.9^c	20.7 ^{c}	21.4^a	21.4 ^{a}	0.1
R7 Line	20.6	18.7^d	21.1^b	20.8 ^{c}	21.6 ^{a}	21.5 ^{a}	0.1

Means with a different superscript letter (a, b, c, d) differ significantly at P < 0.05 level within and between rows for age groups; there is no statistical difference between the pooled flock means.

Table 3. Pearson correlation coefficients (r values) of body weight (BWt), fleece weight (GFWt) and fibre diameter (AFD), staple length (ASL) and comfort factor (CF) characteristics.

	GFWt	FDcv	AFD	ASL	CF
BWt	0.20**	-0.06**	0.21**	-0.13**	-0.10**
GFWt		-0.20**	0.64**	0.45**	-0.36**
FDcv			-0.16**	-0.09**	-0.16**
AFD				0.31**	-0.70**
ASL					-0.13**

**All r values are significant (P < 0.01).

Table 4. Body weight (BWt) at weaning, yearling, 16-month old and fleece weight (GFWt) of the Rafter 7 Ranch sale rams (n = 120/y) (Wuliji et al., 2009).

Year of Birth	Flock ID	Weaning BWt kg	Yearling BWt kg	16 Month BWt kg	10 Month GFWt kg
2006	Merino	27.7	50.9	75.5	3.55
	R7 Line	28.6	52.7	78.6	3.64
2007	Merino	32.7	67.3	74.0	4.09
	R7 Line	37.3	72.7	79.1	4.09
Average/Flocks		31.4	61.4	75.0	3.63

RESULTS AND DISCUSSION

Body weight, GFWt and wool characteristics are shown in **Table 1**. There are significant (P < 0.05) differences between the two selection lines for BWt and GFWt. The pure Merinos produced more wool than the Rafter 7 Line even though their wool was slightly finer. The Rafter 7 Line sheep weighed significantly more (P < 0.05) than the Merino sheep. Over the years of upgrading, the Rafter 7 Line has closed the gap with the Merinos so far as average fibre diameter is concerned while still maintaining a significant advantage in BWt and other subjective traits, such as a larger body frame and structure. But no differences were present between AFD, FDcv, ASL and CF of the flocks. Changes in AFD were shown to exist among birth year (and age) (P < 0.05) (**Table 2**), which reflected the environmental variation, and interaction of birth year and age groups. Pearson correlation coefficients were calculated between BWt, GFWt and wool characteristics and are presented in **Table 3**. There is a significant (P < 0.01) high correlation between AFD and CF, and also high correlations among GFWt, AFD and ASL; but a low and moderate correlation exists between BWt and GFWt, whereas small or negative (P < 0.05) correlations were observed between FDcv and AFD, BWt, GFWt, AFD, ASL, and CF. Weaning weight, yearling weight and GFWt of the Rafter 7 Ranch sale rams have improved over years (**Table 4**). The BWt and GFWt for sale rams of birth year 2006 and 2007 did not differ between the two lines.

Approximately 1000 breeding rams and 500 replacement ewes were distributed to range flocks in the western states in the last decade, which made a notable improvement for GFWt, fibre diameter and yield in clients' flocks (Wuliji et al., 2009). Wool sales from Rafter 7 Ranch have increased significantly in volume and value (**Figure 1**). Sheep flock performance and wool sale information show consistent
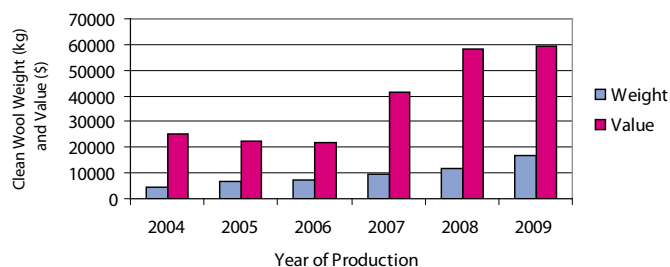
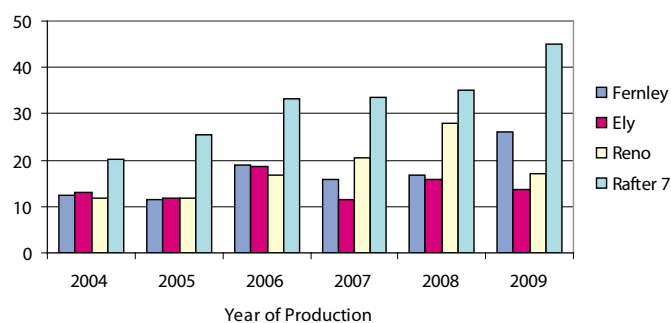**Figure 1. The Rafter 7 Ranch Wool Clip Volume and Value.**

Figure 2. The Superfine Ratio (%) of Wool Clip.

improving trends within Rafter 7 flocks and associated clients' flocks. For the past eight years, the Rafter 7 Ranch wool clip has received the highest price of any wool grown in the USA. Sheep producers from 18 states, Mexico and Canada have purchased breeding rams and ewes from the Rafter 7 Ranch over the past 13 years. The dissemination of introduced Merino genetics of Rafter 7 Ranch into the western range sheep flocks has produced an improvement in wool quality by increasing the superfine wool (<19 microns) ratio of the clip in the associated sheep producer ranches (**Figure 2**). This trend is expected to strengthen a long-term competitive advantage for the western USA states' wool sheep enterprises.

The earlier analysis of Merino crossbred ewes in the flocks showed that clean wool yield, staple length, and GFWt were increased by 15% (67% vs. 52%), 2.5 cm (8.5 cm vs. 6.0 cm) and 1.14 kg/head shorn (5.3 kg vs. 4.2 kg), respectively (Glimp, 2006). Mean GFWt of both Rafter 7 Merino and Rafter 7 Line flocks were apparently higher than published data (Lee et al., 2000) of typical Rambouillet ewes, which were recorded for 4.16 kg/head mixed ages ewes but AFD were lower than the original Rambouillet foundation ewe flocks (25 micron). Body weight measures in sale rams showed a trend that two breeding flocks are converging in terms of for BWt and AFD, although the Merinos produce more and finer wool. The AFD changes by age groups were small to moderate, which is a similar pattern to increase in fleece weight, which showed a larger increase from the first shearing to the second, but small changes until four years of age. Such features of AFD and FDcv, and inter - trait correlations were also observed in ultrafine Merino flocks (Wuliji et al., 1999).

The sheep breeding, wool selection and genetic resource distribution of the Rafter 7 Ranch flock impacts and the breeding stock distribution for western range sheep flocks (Wuliji et al., 2007) and grazing efficiency of ewes on the range were discussed in detail elsewhere (Rauw et al., 2007a). The gradual and continual increase

in superfine and fine wool ratios of the clip at Rafter 7 Ranch was consistent with the early within-flock analysis (Wuliji et al., 2008). Weaning weight of the Rafter 7 flocks were also analysed previously (Rauw et al., 2007b), which showed that ram lambs weighed heavier than ewe lambs, that single reared lambs were heavier than multi-litter lambs, and that lambs born from two-year-old ewes were lighter than from other age group ewes. The selection efficiency in premium wool characteristics and rapid genetic gain were reported for various wool breeding demonstration flocks (Wuliji et al., 1999 and 2001; Swan and Purvis, 2005; Brien et al., 2005). These characteristics showed moderate to high heritability (Atkins, 1997; Okut et al., 1999, Wuliji et al., 2001, Hanford et al., 2004). Therefore, we predict an increased likelihood of a higher rate of genetic dissemination into commercial sheep flocks followed by rapid genetic gains in wool quality traits.

CONCLUSIONS
The Rafter 7 Ranch Merino flocks have made significant progress in major selection traits including fleece weight and fibre diameter during the crossbreeding and upgrading phase. The Ranch is now disseminating elite genetics in many western range sheep flocks. The dissemination of introduced Merino genetics in the western range sheep flocks will improve wool quality and clip profits, thereby strengthening a long-term competitive advantage for the USA wool and sheep production sectors.

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Thai Indigenous Cattle Production Provide a Sustainable Alternative for the Benefit of Smallscale Farmers, Healthy Food, and the Environment

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ABSTRACT

In Thailand, there are 5.66 million Thai indigenous cattle and their crossbred derivatives (1.76 million cows). The Department of Livestock Development (DLD) has a policy to conserve and use Thai indigenous cattle as the genetic base for establishing and developing new breeds of beef cattle. The objectives of this study were to study the production performance, carcass quality, healthy food production, economic potential, and environmental impacts of four breeds of Thai indigenous cattle (Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon cattle). Data were collected from two studies: i) 1 220 cattle from an experimental trial in DLD part, and ii) 390 cattle kept by smallholders in Northern, Northeastern, Central, and Southern parts of Thailand between October 2004 and September 2008. Data were adjusted by group, location, month, and year to analyse for the above parameters. Kho-Lan and Kho-Esarn cattle had the highest weaning weight and preweaning daily weight gains while Kow-Lamphun cattle had the highest Omega 3 (8.98%) and conjugated linoleic acid (CLA) levels in their meat (0.02%), and produced the highest net incomes (306 915.80 Baht/y). Through the conservation and use policies of the DLD, Thai indigenous cattle provided various advantages for farmers, consumers, and environment.

Key words: *Thai, indigenous cattle, weaning weight, carcass, Omega fatty acids, conjugated linoleic acids.*

INTRODUCTION

In Thailand, there are around 5.66 million Thai indigenous cattle and their crossbred derivatives (1.76 million cows) (DLD, 2004). Thai indigenous cattle are classified as *Bos indicus*. Their characteristics are similar to other indigenous cattle in Southeast Asia. The four main breeds of Thai indigenous cattle are Kow-Lamphun, Kho-Esarn, Kho-Lan and Kho-Chon cattle in the northern, northeastern, central and southern parts of Thailand respectively. They are easily raised, selected by natural selection, have good reproductive performance, can provide a calf every year, and are resistant to diseases and para-

sites. Their meat texture is fine and firm and optimised for cooking Thai food. Thai indigenous meat is very tasty and has more specific nutrients that are useful for consumers, such as Omega 3, Omega 6, and CLAs (Boonyanuwat, 2009).

Farmers raise these cattle integrated with other agricultural products, such as rice, para rubber tree, corn, sugar cane, and fish. Their manure is used as fertiliser for crops and producing plankton for fish, and for producing biogas and electric power for household use. In addition, Thai indigenous cattle are used as draught animals. Thai indigenous cattle have various skin and hair colour such as red, light brown, black, piebald, but the Kow-Lamphun cattle in the northern part of Thailand have an orange-pink skin and white hair colour. They are small, heat tolerant, disease resistant, and have high fertility (Boonyanuwat, 2008).

The objectives of this study were to study the production performance, economic potential and impacts on the environment of Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon cattle.

MATERIALS AND METHODS

Animals

Four breeds of Thai indigenous cattle were used in this study:

- Four hundred and eighty eight Kow-Lamphun cattle (370 cattle of DLD, 118 cattle of kept on smallholder farms);
- Four hundred and eighty four Kho-Esarn cattle (367 cattle belonging to the DLD, 117 cattle on smallholder farms);
- One hundred Kho-Lan cattle (76 cattle from the DLD, 24 cattle on smallholder farms); and
- Five hundred and thirty eight Kho-Chon cattle (408 cattle from the DLD, 130 cattle on smallholder farms).

These four breeds were separated roughly into two groups (a cow-calf production group and a finishing group). They were fattened at DLD Research and Breeding Centres/Stations and on smallholder farms (Kow-Lamphun cattle in Lamphun, Chiangmai, Phayao, and Phrae provinces; Kho-Esarn cattle in Ubolrachathani and Chaiyaphum provinces, Kho-Lan cattle in Ratchaburi and Petchaburi provinces; and Kho-Chon cattle in Phatthalung, Trang, Songkla, and Yala provinces) between October 2004 and September 2008.

Measurements and Statistical Analysis

Data from the DLD groups were collected every six months while data from smallholder farms were collected every month. Data were collected on growth performance (bodyweight (BWt), average daily gain

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(ADG)), carcass performance (carcass %, loin eye area, shear force, fatty acid profile), economic performance (net income per head) and cattle manure production. Samples for shear force and fatty acid profile measurement were collected from the 7th–8th rib.

The samples and data for carcass performance were collected at the Research and Breeding Center/Station of the DLD, Kampangsaen slaughter house of Kasetsart University and the Central Laboratory of Chiangmai University. Data were adjusted for variations arising from group, location, month, and year and the adjusted data were analysed by ANOVA for growth, carcass and economic performances (Chantalakhana, 1991).

RESULTS AND DISCUSSION

Growth and Reproductive Performance

The average bodyweights of each breed were not significantly different. Kho-Lan cattle had the highest weaning weights (WWt) and ADG (103.18 kg and 0.421 kg) (**Table 1**). In the DLD group, they were fed with grass and legume. They were raised by grazing in pasture *(Bachiaria ruziziensis* and *Stylosanthes hamata)*. In the farmers groups, they were fed with grass, or grass and legume, and supplemented with concentrates after calving. Kho-Chon cattle had the lowest WW. In southern Thailand, farmers prepare male calves for use as fighting bulls. There were no significant differences in reproductive performances. Thai indigenous cattle can give birth every year with calving intervals of 433 to 469 d.

Carcass Performances and Meat Quality

The meat texture of the four breeds was not significantly different. It is very firm (shear force = 5.45-5.56) (**Table 2**) and suitable for Thai food cooking. Meat from these cattle is used to make meat balls and Thai food.

Kow-Lamphun cattle had the highest Omega 3 fatty acids (8.98%). However, Omega 3 levels in all indigenous breeds were high (Kho-Lan = 6.25%, Kho-Chon = 6.26%, and Kho-Esarn = 2.37%) compared with Bos taurus (2.90% for grass fed and 0.64% for grain fed animals (Daley et al., 2009). Omega 3 beef was different from typical beef in that it was obtained by grass feeding while typical beef is most often obtained through grain feeding. Grass was higher in Omega 3 fatty acids while grains that were fed to animals were higher in Omega 6 (Helmet, 2009). Even so, Thai indigenous cattle

that were grain fed had high Omega 3 levels (e.g. Kho-Esarn, grain fed and grass fed).

Native cattle had the highest Omega 3, Omega 6, and polyunsaturated fatty acid (PUFA) levels. Conversely, Kow-Lamphun cattle had the lowest Omega 6 levels (3.67%). Kho-Chon, Kho-Lan, and Kho-Esarn had higher Omega 6 (4.36, 5.48, and 10.14% respectively). Thus, the ratio of Omega 6:Omega 3 of Khow-Lamphun cattle was lowest, next to Kho-Chon, Kho-Lan, and Kho-Esarn. The recommended ratio of Omega 6 to Omega 3 fatty acids is 2:1 or better. Omega 3 beef is also loaded with natural vitamins and minerals. It is a great source of CLA (conjugated linoleic acid), a fat that is reputed to reduce the risk of obesity, cancer, diabetes, as well as some immune disorders. Beef in its natural state and grass fed, not grain fed, allows it to be categorised as a health food. This is a red meat that is actually good for consumers (Helmet, 2009).

Thai indigenous cattle meat was very rich in Omega 3 fatty acids as well. It is also free from hormones and antibiotics. A proper balance between Omega 3 and Omega 6 fatty acids helps to maintain and even to improve health. A healthy diet should consist of roughly one to four times more omega 6 fatty acids than omega 3 fatty acids (Daley et al., 2009).

Kow-Lamphun and Kho-Lan cattle had higher CLA levels than Kho-Chon and Kho-Esarn cattle, 0.02, 0.02, 0.01, and 0.01, respectively (P < 0.01). CLAs are a group of polyunsaturated fatty acids found in beef, lamb, and dairy products consisting of a general mixture of positional and geometric conjugated isomers of linoleic acid (Sehat et al., 1999). They are produced in the rumen of cattle and other ruminants during microbial biohydrogenation of linoleic and linolenic acids by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens* (Pariza et al., 2000). Over the past two decades numerous health benefits have been attributed to CLA in experimental animal models including actions to reduce carcinogenesis, atherosclerosis, the onset of diabetes, and fat body mass (Daley et al., 2009).

The anti-atherosclerotic evidence was first reported in CLA treated mice by Ip et al. (1994). Ip and co-workers showed that CLA levels as low as 0.05% of the diet can have a beneficial effect in mice. A level of 0.5% reduced the total number of mammary tumours by 32%. These results also demonstrated that CLA administered through a dietary route was effective in providing protection against cancer (Ip et al., 1994). However, there is high CLA in Thai indigenous cattle meat.

Although there were no significant differences between indigenous breeds in PFU levels, these levels were high compared with

Table 1. Growth and reproductive performances of Thai indigenous cattle.

Trait		Kow-Lamphun (n = 488)	Kho-Esarn (n = 484)	Kho-Lan (n = 100)	Kho-chon (n = 538)
BWt	(kg)	19.64 ± 2.86	18.38 ± 5.34	18.57 ± 2.58	19.91 ± 1.40
WWt**	(kg)	90.22^b ± 29.12	100.38^a ± 10.51	103.18^a ± 14.32	87.81^b ± 12.04
ADG**	(kg/day)	0.353^b ± 0.117	0.410^a ± 0.053	0.421^a ± 0.059	0.340^b ± 0.052
BWt 400**	(kg)	204.30^b ± 39.11	221.55^a ± 42.92	210.71^a ± 29.25	198.45^b ± 27.21
BWt 600*	(kg)	296.04^b ± 39.48	323.14^a ± 64.38	314.54^a ± 43.66	296.54^b ± 58.52
Age first calving	(month)	28.34 ± 4.43	26.47 ± 7.69	26.92 ± 3.74	28.67 ± 2.02
Calving interval	(d)	463.67 ± 117.24	433.00 ± 125.80	448.59 ± 62.27	469.04 ± 33.05

BW = birth weight; WW = weaning weight; ADG = preweaning ADG; BWt 400 = body weight at 400 d of age; BWt 600 = body weight at 600 d of age.

** Different letter in the same row means highly significant difference of means in each trait (P < 0.01).

* Different letter in the same row means significant difference of means in each trait (P < 0.05).

Table 2. Carcass performances and healthy food production of Thai indigenous cattle.

Trait	Kow-Lamphun (n = 34)	Kho-Esarn (n = 16)	Kho-Lan (n = 23)	Kho-chon (n = 36)
Final weight (kg)**	$320.47^a \pm 44.49$	$299.21^b \pm 41.5$	$302.33^b \pm 41.97$	$323.49^a \pm 44.91$
ADG (kg/d)	0.668 ± 0.79	0.624 ± 0.447	0.630 ± 0.452	0.674 ± 0.484
Carcass (%)	56.94 ± 10.03	54.11 ± 9.37	54.66 ± 9.46	58.49 ± 10.12
Loin eye area (cm^2) **	$57.46^a \pm 6.34$	$53.67^b \pm 5.92$	$54.21^b \pm 5.98$	$58.01^a \pm 6.40$
Shear force (kg) (21 d)	5.49 ± 0.57	5.50 ± 0.54	5.56 ± 0.54	5.45 ± 0.58
Omega 3 (% total fat)**	$8.98^a \pm 7.55$	$2.37^c \pm 0.69$	$6.25^b \pm 5.02$	$6.26^b \pm 1.87$
Omega 6 (% total fat)**	$3.67^c \pm 0.57$	$10.14^a \pm 4.74$	$5.48^b \pm 3.47$	$4.36^b \pm 1.54$
CLA (% total fat)**	$0.02^a \pm 0.02$	$0.01^b \pm 0.01$	$0.02^a \pm 0.01$	$0.01^b \pm 0.01$
SFA (% total fat)**	$80.27^a \pm 9.37$	$49.45^c \pm 3.27$	$72.59^b \pm 14.94$	$82.27^a \pm 4.51$
MUFA (% total fat)**	$6.78^c \pm 6.38$	$37.85^a \pm 5.89$	$14.42^b \pm 4.29$	$6.83^c \pm 3.63$
PUFA (% total fat)	12.94 ± 7.99	12.70 ± 5.45	12.00 ± 5.23	10.90 ± 2.04

CLA = conjugated linoleic acids; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

** Different letter in the same row means highly significant difference of means in each trait (P<0.01).

Table 3. Economic performances and impacts on environment.

Trait		Kow-Lamphun (n = 3)	Kho-Esarn (n = 15)	Kho-Lan (n = 3)	Kho-chon (n = 30)
Farm size	(No. cows)	$18.00^a \pm 5.18$	$3.80^b \pm 1.86$	$19.30^a \pm 7.81$	$2.94^b \pm 1.07$
Age of cow	(y)	4.42 ± 1.84	4.50 ± 1.87	4.88 ± 1.58	4.85 ± 1.25
Total cost	(Baht)	$54\,802^b \pm 13\,700$	$24\,950^d \pm 8912$	$12\,6746^a \pm 11318$	$26\,768^c \pm 13\,859$
Feeding cost	(Baht)	$28\,597^b \pm 7149$	$17\,217^c \pm 4054$	$8\,7464^a \pm 5149$	$13\,451^c \pm 1\,118$
Total income	(Baht)	$364\,718^a \pm 91\,179$	$57\,628^c \pm 9616$	$29\,2751^b \pm 12213$	$23\,161^d \pm 18\,253$
Net income	(Baht)	$915^a \pm 76728$	$32\,678^c \pm 704$	$16\,6006^b \pm 3579$	$7\,838^d \pm 4\,257$
Fertiliser from manure	(ton)	$75.88^a \pm 1.04$	$16.02^b \pm 1.49$	$81.36^a \pm 3.78$	$12.39^b \pm 0.86$

** Different letter in the same row means highly significant difference of means in each trait (P<0.01).

other breeds and grain fed beef cattle (levels in Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon averaged 12.94, 12.70, 12.00, and 10.90%, respectively, **Table 2**). Nonetheless, intramuscular fat of other beef breed and grain fed typically consisted of approximately 47, 42 and 4% of total fatty acids as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA, respectively. The PUFA in beef contains considerable amounts of n-3 PUFAs, particularly α-linolenic acid (C18:3n-3) and the longer chain PUFAs, eicosapentaenoic acid (EPA; C20:5n-3) and, docosahexaenoic acid (DHA; C22:6n-3) (Scollan, 2009).

Two important nutritional indices are used to describe the fatty acid composition of foods. The first is the ratio of PUFA:SFA (the P:S ratio), and the second the ratio of the n-6:n-3 fatty acids (usually expressed as the ratio of essential fatty acids C18:2n-6 (linoleic acid): C18:3n-3 (linolenic acid). The P:S ratio for beef is typically low at around 0.1, except for double muscled animals which are very lean (<1% intramuscular fat) where P:S ratios are typically 0.5-0.7. Results from EU Healthy Beef have demonstrated a strong relationship between total intramuscular fat content and P:S ratio (Scollan, 2009). The ratio of n-6:n-3 ratio for beef is beneficially low, typically less than 3 and the focus has been on methods of increasing the P:S ratio and lowering the n-6:n-3 ratio by increasing the content of beneficial n-3 PUFA (Scollan, 2009). Kow-Lamphun cattle had a high P:S ratio.

Economics and Environmental Impact

From the 51 smallholder farms used to collect data, the average herd sizes of Kow-Lamphun, Kho-Esarn, Kho-Lan, and Kho-Chon, were 18.00, 3.80, 19.30, and 2.94 cows respectively (**Table 3**). The data for Kow-Lamphun and Kho-Lan cattle were obtained only from conservation farms, while others were collected from general farm. On the conservation farms, farmers could earn higher net incomes. In these two farms, farmers could decide on the price for conservation and rare animals. These farms could produce fertiliser from manure to provide organic material for the land (**Table 3**). On the basis that there are 5.66 million Thai indigenous cattle, the data on manure production recorded here suggests that these animals could contribute 177 000 tons of dry manure. In this way, Thai indigenous cattle improve the structure of land and thereby enhance the environment.

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Community-Based Productivity Veterinary Service for Smallholder Dairy Farmers in Bangladesh

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ABSTRACT

The productivity veterinary services, which include disease control and management of reproduction, udder health and nutrition, are not practised in smallholder dairy farms although they are proven to increase milk production in large dairy herds. We introduced an on-farm service with the participation of farmer associations where individual veterinarians made a scheduled visit to perform preventive and emergency cattle health care, reproduction, and feed management. We examined 1 849 animals on 862 farms guided by specific forms, a breeding calendar and a herd summary generated from data of the initial visit by using a Microsoft Access based computer application. On average, 53% anoestrous heifers and 67% anoestrous cows resumed their oestrous cycle when treated with hormones, vitamin AD_3E or nutritional supplements. Forty percent of cows with uterine infections conceived when treated with intrauterine antibiotics or prostaglandin $F_2\alpha$ ($PGF_2\alpha$) was injected intramuscularly before artificial insemination (AI) was done. When GnRH was injected at the time of AI, 73% repeat breeder cows conceived. About 78% of cows recovered from mastitis and 88% of sick animals recovered when treatment was given based on clinical diagnosis. A database on common cattle diseases was established. More than 75% of farms that received the service had an income increase ranging from US$1 to US$40.7/month/cow. Productivity veterinary services can increase farmers' incomes and the number of cows available for breeding.

Key words: *productivity veterinary services, smallholders, health care, reproduction, feed management, anoestrous, uterine infections, artificial insemination, income increases.*

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INTRODUCTION

Bangladesh needs to improve the growth of its dairy industry from the current rate of two percent to at least six percent to meet half of the consumer demand for milk by the year 2025 against a population growth rate of 1.6%. Farmers' incomes would increase by between US$676.3–1 730.6/y if all of them operated their farms as efficiently as the 20% best farmers in the community concerning levels of milk production/cow/d, increasing lactation length, decreasing age to first calving, and decreasing calving interval (Shamsuddin et al., 2006). Farmers spend only a small amount of money on veterinary services (Shamsuddin et al., 2006), and the benefits of veterinary services on the productivity of animals have shown a poor return on investment. Veterinarians neither have information on the private job market nor do they have a model for delivering the service and recovering the cost. The biggest challenge is that farms are too small and farmers cannot buy the service individually. An alternative approach of working through farmers' associations would be useful to execute and finance a market-driven veterinary service. We report here a model of delivering productivity veterinary services to smallholder dairy farms through farmers' groups and associations which would substantially increase their income.

MATERIALS AND METHODS

Farms, Farm Sizes and Production Systems

In the popular areas for small scale dairy farming in the districts of Satkhira, Mymensingh, Chittagong and Sirajgonj, about 250 farms were selected in each and divided into groups of ten farms. One farmer in each group worked as the Group Leader. One veterinarian following a previously set schedule visited ten farms each d every month, with the Group Leader being kept informed. Thus during the 25 working d of a month, the veterinarian visited 250 farms. Twenty five Group Leaders made an association. Data reported here were from four of such associations constituting 1 000 farm families during the period March 2005 to June 2006.

The dairy production systems in these districts were published elsewhere (Shamsuddin et al., 2006 and 2007). Individual farms in Satkhira had an average of six cattle with 2.2 lactating cows. Individual farms in Mymensingh had 4.2 cattle with 1.4 lactating cows. The production system was crop-livestock mixed farming in Mymensingh and Satkhira. The dairy farmers of Sirajgonj had access to the Bangladesh Milk Producers' Cooperative Union Ltd. and they were heavily dependent on dairying for their livelihood. The average

number of cattle/farm was 10.5 with 3.4 lactating cows. Dairy farming is commercial and intensive in Chittagong. Here, the average numbers of total cattle and lactating cows/farm were respectively 8.0 and 2.8.

Collection of Information, and Provision of Veterinary Services and Follow-up

To guide delivery of the veterinary service and to follow up on its outcome and collect field data, we developed five forms (Shamsuddin et al., 2009). A calendar was developed to keep records on breeding, vaccination, de-worming and other health and production-related events in the farm.

Form 1

This form had four parts dealing with issues such as a farm inventory, preventive health management, feed management and a list of animals treated for sickness. Data on existing animals on the farm, animal types and their production were recorded. If any animal was sold, its type and price was recorded. If an animal died, its type was recorded. Information on de-worming, vaccination, teat dipping and examination of fore milk was recorded. The date of drug administration, its cost and administration fees were recorded with regard to the type of the animal. For feed management, at first the amount of feed given to animals was recorded together with the feed price. If necessary, changes in feed composition and amount were recommended and recorded in the form. The identity of individual animals treated and herd nutrition conditions were also recorded, the latter on the basis of a 1–5 scale (Nicholson and Butterworth, 1986).

Form 2

This form helped recording information on breeding cows and heifers, cost of breeding and pregnancy diagnosis. The form guided recording farmers' complaints and taking previous histories on e.g. parturition, puerperium, retained placenta, number of services used for last conception and milk production of cows with reproductive problems. Animals were examined and temperatures, heart rates, respiratory rates, rumen contractions-frequencies and strength and nutrition conditions were recorded. Genital tracts and ovarian cyclicity were evaluated by inspection and rectal examinations. In follow-up visits, results of treatment were determined and further necessary interventions adopted.

Form 3

This form allowed recording data on mastitis diagnosis and treatment. Farmers' complaints and animal history were recorded. In addition to physical examinations of cows and their udders, milk was examined by visual inspection and by the California Mastitis Test (CMT). Findings of the examinations were scored and recorded. Mastitis was diagnosed and its severity was graded as mild, moderate and severe based on scores given through examinations of the cow, udder and milk. The form had a guideline for the treatment or management of mastitis, and options for recording the outcomes of treatments at follow-up visits.

Form 4

This form was designed to guide examination of sick animals other than those with reproductive problems and mastitis. Farmers' complaints and animals' history of sickness were recorded. They were examined for heart rate, respiration rate, rectal temperature and rumen contraction-frequency and strength, consistency of faeces,

hydration, appetite and nasal conditions for making a clinical diagnosis. Prescribed treatments and their costs were recorded in the form. The form had provision for recording the results of treatments at follow-up visits.

Form 5

This form was used to record all expenditures and incomes of the farm operation on a monthly basis. Incomes from milk and home use of milk, manure sales and/or home use, sales and slaughter of livestock and costs for feed purchase and freight, health care, labour and maintenance were recorded.

A Microsoft Access-based database application was customised, matching with the forms to record and analyse the data and to produce a herd summary. At farm visits, the veterinarian checked the results of earlier interventions and schedules of de-worming and vaccination. The veterinarian then checked the breeding calendar for reproductive events and especially examined cows bred 35 or more d earlier for pregnancy, cows that gave birth 60 or more d before for ovarian cyclicity, and cows that failed to conceive after three consecutive services. A clinical diagnosis was made and treatment and/or management changes were prescribed. Heifers that were more than two years old but had not shown oestrus were examined for ovarian cyclicity. The veterinarian also looked at the drying-off date of the cow, milking hygiene and post-milking teat dipping. Additionally, the farmer could call a veterinarian if any emergency or general cattle health care issue arose on the farm.

Data Management and Analysis

Data from the customised database application were exported to a Microsoft Excel Workbook 2002. Only farms that accepted the interventions were included. Descriptive statistics were computed and histograms drawn. Regression analysis was done to establish the relationship between the number of lactating cows on a farm and the net income from the operation. Farms from Satkhira and Mymensingh that had data from at least three visits were included for the analysis of economic impacts of interventions. Net income (gross income - gross cost) was calculated/cow/d for before and after interventions. The information obtained on the first visit was considered as pre-intervention data, while data from subsequent visits were averaged to calculate post-intervention values. The initial net income from individual farms was deducted from the post-intervention net income to determine changes in farmers' income from the service.

Considering the possible influence of flush and lean season on the economic impact of the service in Sirajganj, additional data on expenses and incomes from dairy operation were collected through one-day farm visits by using preset questionnaires from 204 member farms and 60 non-member farms. Non-member farms did not receive the service but were situated in the same locality where interventions were performed. Data were entered into Microsoft Excel Worksheet 2002, total incomes and expenditures/year/farm were estimated individually for intervened and control farms, returns calculated and histograms prepared. Differences in returns between intervened and control farms were tested for significance using the z-test.

RESULTS

We examined 1 849 animals on 862 farms. In follow-up examinations, 53% anoestrous heifers and 67% anoestrous cows resumed their oestrous cycle when treated with preparations of hormones, vitamin AD_3E or with nutritional supplementations, on average. Forty percent of cows with uterine infections conceived when antibiotic was given into the uterus or $PGF_2\alpha$ was injected intramuscularly
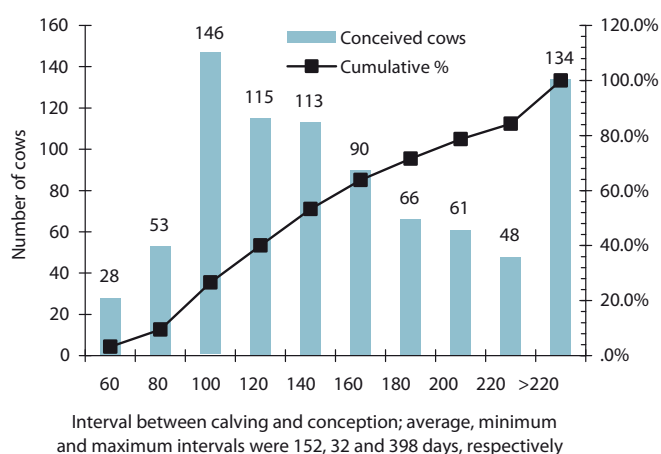
Figure 1. Number of cows and their cumulative percentages conceived at different days postpartum (number of cows = 854).

before AI was performed. When gonadotrophin-releasing hormone (GnRH) was injected at the time of AI, 73% of repeat breeder cows conceived. About 78% of cows with mastitis cows recovered after treatments and 88% of sick animals recovered when treatment was given based on clinical diagnosis.

Reproductive Management

In total, 914 cows and heifers were examined for pregnancy diagnosis by rectal palpation and 854 were pregnant (**Figure 1**). Sixty cows (6.6%) were found non-pregnant. Only 25% of cows conceived within 100 d of calving and 44% of cows took 160 to 398 d postpartum for conception. Farmers paid between US$0.6 and US$15.4 for breeding an animal to achieve a pregnancy.

Management of Anoestrous Cows and Heifers

In total, 389 cows and 279 heifers were diagnosed as anoestrus and prescribed treatments. Follow-up data were available on 93 cows and 101 heifers. On average, 71% of cows and 53% of heifers resumed oestrous cycles after treatment (**Table 1**). Farmers considered 35 cows (9%; n = 389) anoestrus but those had a corpus luteum at

their ovaries (**Table 2**). These cows were treated with PGF2α with or without GnRH and inseminated either at detected oestrus or blindly at a fixed time i.e. 70 and 90 h after PGF2α injection. Eighty percent of cows were presumed pregnant (non-return by d 30 or diagnosed pregnant). The cost of treatment for individual animals varied from US$1.5–17.0. The overwhelming majority of animals (87%) were treated for US$1.5–3.7.

Management of Cows and Heifers that Failed to Conceive after Three Services

The veterinarian examined 118 cows with a history of conception failure after more than three services and prescribed treatments accordingly. Follow-up data were available on 56 cows. Forty animals were treated with GnRH preparations at the time of AI and 29 of those (73%) became pregnant. Sixteen animals were inseminated twice on the same oestrus at 12 h intervals; 12 of these conceived. The cost of treatment ranged from US$1.5–8.8 per cow.

Management of Cows with Infected Uterus

Fifty five cows with uterine infections of varying degrees — ranging from cloudy genital discharge to discharge of pus — were diagnosed and treated. The treatment was either intramascular injection of a prostaglandin F2α preparation or intrauterine application of antibiotics. Data on 30 cows were available on follow-up visits. Twelve (40%) of cows conceived, and the cost of treatment ranged from US$1.5 – US$4.4/cow.

Mastitis Cow Management

One hundred and fifty one cows with complaints of udder problems were examined and treatments were prescribed. Twenty eight of 36 cows showed normal udder and milk on follow-up visits (**Table 3**). The treatment cost for mastitis ranged from US$2.9– 8.8/cow.

General Cattle Disease Management

Three hundred and ninety five animals were examined and treatments were prescribed. Follow-up data were available on 187 animals. Most of the treated animals recovered from illness and only two animals died (Table 4). Frequency percentages for the different disease conditions are shown in Figure 2. The overwhelming majority of clinically diagnosed diseases were diarrhoea/dysentery (18.5%) and endoparasites (20.5%). The treatment cost for general disease

Table 1. Treatments with outcomes of anoestrus cows and heifers.

Treatment used	Number of animals treated		Number (%) of animals cycled	
	Cows	Heifers	Cows	Heifers
[1] GnRH (+ PG + AI + GnRH)	14	12	12 (86)	7 (58)
Anthelmintics + ADE	35	28	26 (74)	16 (57)
ADE	22	49	14 (64)	23 (47)
Nutrition supplement	22	12	14 (64)	8 (67)
Total	93	101	66 (71)	54 (53)

[1] Prostaglandin F2α was injected 12 d after GnRH injection and AI was done on oestrus with an additional GnRH injection at the time of AI; GnRH — commercially available synthetic gonadotrophin-releasing hormone preparations; PG = prostaglandin F2α preparations available commercially; AI — artificial insemination; ADE — an injectable preparation of vitamin A, D3 and E available commercially.

Table 2. Treatments with outcomes of cyclic cows that farmers considered anoestrous.

Treatment used	Number of cows treated	Number of cows non-returned or pregnant
PGx1+AI on oestrus	9	8
PGx2+2 times AI at 12 h interval	7	6
PGx2+2 times AI at 12 h interval + GnRH with first AI	10	7
GnRH (+PG+AI+ GnRH)	5	4
2 times AI at 12 h interval at detected oestrus	4	3
Total	35	28

PG — prostaglandin F2α preparations available commercially; GnRH — commercially available synthetic GnRH preparations; AI — artificial insemination.

Table 3. Treatments with outcomes of cows with mastitis.

Treatment used	No. treatments	No. positive responses
Intramammary antibiotics only	15	13
Intramammary antibiotics with parenteral administration of anti-inflammatory drugs	7	4
Intramammary antibiotic + anti-inflammatory drugs + antibiotics administered intramuscularly	12	10
Systemic antibiotic only	2	1
Total	36	28

Table 4. Number of animals examined, treated and followed up with results of treatments.

Parameters	Number	Percentage
Cattle examined and treatment given	395	
Cattle followed up	187	47.3
Animals completely cured	165	88.2
Animals showing some improvement	13	7.0
Animals showing no improvement	7	3.7
Animals that died	2	1.1

conditions ranged between US$0.7 and US$2.9. A database was made of cattle health problems (Figure 2).

Effects of Productivity Veterinary Service on Net Incomes of Farmers

Changes in farmers' net incomes due to the services delivered in Satkhira and Mymensingh are shown in **Figure 3**. In Satkhira, more than 75% of farms had an increase in net income, which ranged from US$1.0 – US$19.2/cow/month. In Mymensingh, more than 80% of farms had increased income due to the productivity veterinary services; increases ranging from US$1.0 – US$40.7. In Sirajganj, farmers that received the service had a higher (P<0.05) return than those who did not (**Figure 4**).

When data from all farms were pooled, a significant relationship appeared between the number of lactating cows on a farm and the net income (**Figure 5**; P < 0.05). Farms that incurred a loss had four or fewer lactating cows over the intervention period.

DISCUSSION

Community-based productivity veterinary services increased incomes in the majority of dairy farms. While the positive effects of herd health management or productivity veterinary services were demonstrated elsewhere (Dijkhuizen et al., 1984), reports on such programmes in smallholder dairy farms are scanty (Suriyasathaporn and Singhla, 2008).

Some farmers did not achieve income increases from our services. Firstly, one-year interventions on cattle health and reproduction may not necessarily produce an impact on farmers' incomes because in many cases the treated animals are not in production. Secondly, there were farms in this study where a lactating cow was not always present. Farmers with too few cows face difficulties in sustaining a return that is more than US$1.0 (**Figure 5**). Since milk is the major source of income on a dairy farm, small farms without a lactating cow generate no income but costs are incurred in feeding the animals. Providing veterinary services in such situations will not produce an immediate visible economic impact.
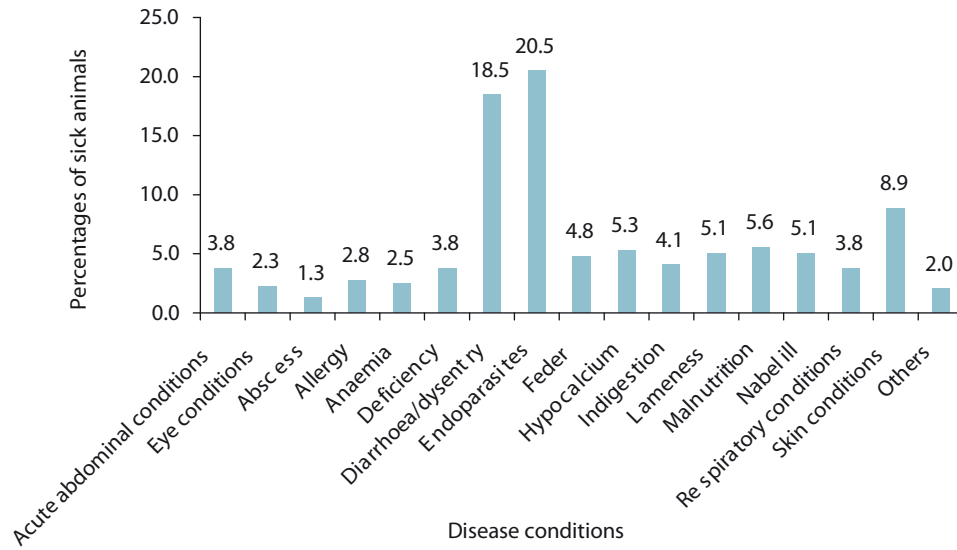
Figure 2. Frequency percentages for general disease conditions of cattle in Bangladesh (n = 395).
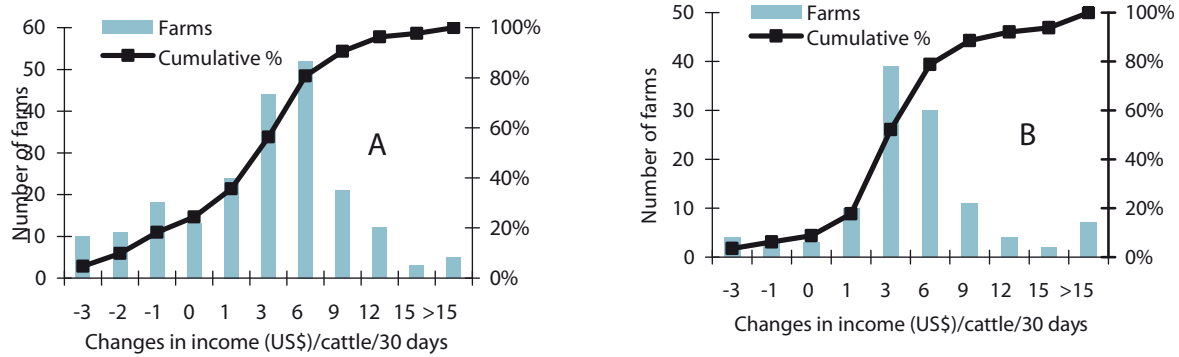
Figure 3. Effects of productivity veterinary services on farmers' net incomes. In Satkhira (A), minimum and maximum differences were US$ –8.0 and US$19.2 (number of farms = 213); in Mymensingh (B), the differences were US$ –8.4 and US$40.7 (number of farms = 114).
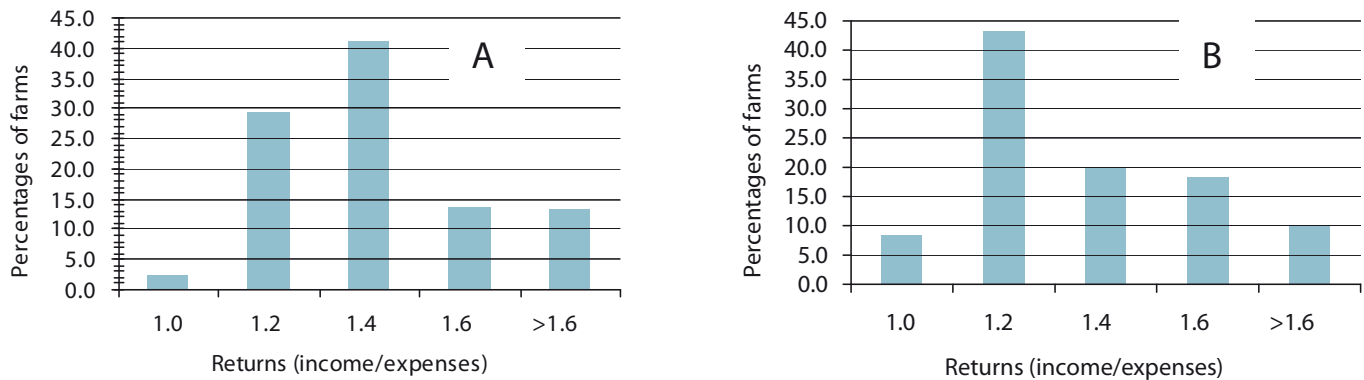
Figure 4. Distribution of returns. A = 204 farms that received the productivity veterinary service; B = 60 control farms.
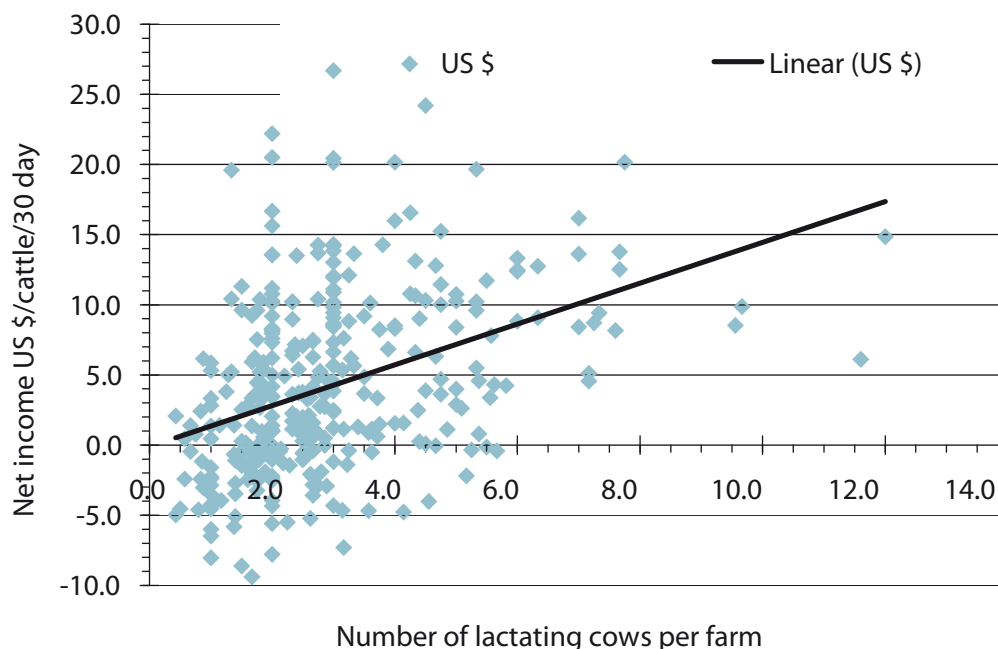
Figure 5. Relationship between number of lactating cows/farm and average daily net income/cow (number of farms = 332).

Thirdly, results of this study show that the method used for economic evaluation does matter in revealing the impacts of interventions in dairying. For example, Sirajganj has a clear flush and lean season for milk production (Shamsuddin et al., 2007). If the service starts at the flush season when the control data are taken and continues through the lean season when milk production is low, it is unlikely that the effect of intervention will be demonstrated.

The forms and calendar developed in this study proved useful in obtaining data consistently from smallholder dairy farm families. The database application was helpful for analysing the data collected from the field services and producing a herd summary for guiding the interventions in follow-up visits

Bangladesh and many other countries in South East Asia have farms with as few as one cow. Therefore, the herd health veterinary service delivery models available in countries with developed dairy industries may not be feasible for these situations. The challenges include the service-purchasing capacity of individual farmers, utilisation of veterinarian's time, time spent travelling from farm to farm, veterinarian's ability to adopt state-of-the-art technology and their unfamiliarity with such systems. The idea of delivering the service through farmers' associations proved useful in this pilot study. However, further studies are required to demonstrate the feasibility of replication and scaling-up.

Routine examination of cows by following a pre-scheduled farm visit identified cyclic cows which otherwise would have been considered non-cyclic by the farmers. Simple treatment with prostaglandin that incurs a reasonable cost allows breeding these cows with a good chance of resulting pregnancies.

A considerable proportion of cows and heifers that were identified anoestrous responded to treatment with GnRH or a mixed commercial preparation of vitamin A, D3 and E. Generally, the responses of anoestrous cows to GnRH treatment are poor since such treatment requires the presence of a functional ovarian dominant follicle in the ovary (McDougall et al., 1995). The good response in this study

could be due to (a) the cows being quite late in their postpartum periods, (b) many of these cows could have been cyclic since a poor heat detection exists on the farm or (c) perhaps farmers paying more attention to the cows once diagnoses were made and costly hormone treatment was given. Whether rectal palpation of ovaries stimulate cyclicity in anoestrous cows and prepubertal heifers remains to be investigated. Sometimes postpartum anoestrous cows do not show oestrus within the expected period after GnRH administration but treated cows subsequently demonstrate a reduction in the postpartum anoestrous period (Khair, 2005).

In anoestrous heifers, the GnRH treatment did not add any benefit over de-worming, vitamin-mineral administration and/or nutritional supplementation. This again supports the beneficial effects of nutrition on reproduction. Whether any metabolite of gastrointestinal parasites has a negative effect on ovarian cyclity or the removal of parasites makes more nutrients available to animals needs to be examined.

In this study, clinical mastitis responded well to antibiotic treatment. However, more specific treatment guided by the results of milk culture for bacteria would hasten recovery of animals and reduce the risk of undue milk contamination with drug residues. The preventive programme for bovine mastitis in Bangladesh is quite new and currently we are running a project to support mastitis management based on information from milk culture for bacteria.

Common cattle health, reproduction and production-related problems were identified by this study, and the costs of different veterinary interventions are known. This will help in designing private, on-farm productivity veterinary services with the means of recovering costs to run the programme. A future opportunity would be a foundation of veterinarians that would work hand-in-hand with producers' associations for institutionalising a private veterinary service in Bangladesh.

In conclusion, the community-based productivity veterinary service increased the incomes of the majority farmers and the number of

cows breeding in farms; however, it needs to be further strengthened through consistent farm visits and institutionalising the service.

ACKNOWLEDGMENTS

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Managing Livestock in Degrading Environments

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ABSTRACT

Degraded environments are both widespread (being found on all continents on earth) and diverse. They have been broadly classified as: irrigated (and rain-fed) farmland with elevated water tables causing salinity; rain-fed farmland with soil erosion, loss of organic matter, nutrient depletion and weed invasion; and degraded rangeland. This review considers all these but with a focus on the first two, and particularly addresses options for simultaneous improvement in livestock production and landscape health. There is evidence that responsible grazing is consistent with ecosystem benefits and resilient land use systems; exclusion from grazing may reduce diversity and create management complexity. Responsible grazing however will only prevail if the land owner or user receives a financial benefit in the process. Solutions need to be profitable. In the development and management of grazing systems, expectations need to be realistic. The prescriptive approach to livestock feeding based on the selection and cultivation of a small range of improved plant species to meet predetermined energy, protein and mineral requirements is inappropriate. Degraded landscapes are often associated with a high edaphic and climatic variability that is best suited to a diverse range of plant species in an assembly that will fluctuate over time and space. This diversity means that under some circumstances degraded land may contribute to reduced risk within a whole farm business. Simultaneous objectives for livestock and landscape improvement may or may not contribute to the return of the landscape to its original state. In some cases stable vegetation that provides some of the functional benefits of the original landscape, such as improved biodiversity and soil health, combined with production benefits is the best option available. This provides an opportunity to establish a range of objectives in vegetation management and design. In Australia, such an approach is leading to the development of new farming systems that use salinised and degraded cropland for livestock. Livestock can cope with the diversity of vegetation that is suited to degraded landscapes; they have the ability to select a diet based on the minimisation of metabolic cost. They not only optimise energy and protein intake but select combinations that increase their ability to deal with toxins and parasites and to modify metabolic processes. This does not necessarily mean they will thrive; low biomass production cannot be overcome by increased choice alone, but it does mean we may need to learn from animal behaviour rather than endeavour to control it. With limiting biomass, complementary and supplementary feeds may still be required to improve the efficiency of use of grazed plants or to manipulate grazing where degradation is concentrated. There are also opportunities for strategic revegetation with plants selected for a range of nutritional, medicinal and ecosystem benefits. Just as plant species that have been bred for highly productive systems are usually inappropriate for degraded environments, so too are livestock. Traditional breeds may be better able to cope with the diverse feeding options, difficult terrain and variable climate and be more efficient in energy use. Animals bred for high production systems often partition a high proportion of available nutrients to production when feed supply is abundant but store less nutrients and are therefore less able to survive and reproduce during periods of low feed availability. Breeding within the relevant environment also exposes animals to stressors in utero and this may improve their ability to cope with these in later life. The concept of responsible management depends on available labour or technology for monitoring of both livestock and environment. Technology is now available or under development that will allow monitoring of livestock condition and detailed information on behaviours. These parameters are closely related to the condition of the grazing environment; the animal acts as a natural integrator of the information that describes the environment. This sensitive direct feedback mechanism is very powerful and offers new opportunities in the simultaneous management of livestock and the environment. In conclusion, degrading environments provide an opportunity for the profitable production of food. Livestock systems may be designed to retrieve or sustain landscape functionality. Livestock systems management within these environments requires an innovative approach that integrates the skills of animal physiology and behaviour, agronomy, plant ecophysiology, soil science and ecosystem ecology and management. This integration must operate outside the narrow perspectives that often characterise these disciplines.

Key words: *degraded environments, livestock productivity, landscape health, grazing management, plant diversity, genotype and phenotype selection, remote monitoring.*

INTRODUCTION

There is no shortage of publications on grazing livestock in degrading environments. Some of these address the landscape and how to protect it from livestock with limited consideration of the metabolic consequences for the grazing animal (Ash and McIvor, 2005). Others focus more specifically on the nutrition and feeding strategies of ruminants with less emphasis on the condition of the natural resource (Ben Salem and Smith, 2008). This paper primarily addresses options to improve both livestock and landscape health simultaneously. It is about how forage grown in degrading environments can supply the needs of grazing animals and, at the same time be managed to reduce or reverse degradation.

Kassas (1995) describes the three major land use types that are susceptible to degradation as:
- irrigated farmland with elevated water tables causing salinisation and soil damage;

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- rain-fed farmland with soil erosion, loss of organic matter and nutrient depletion and weed invasion; and
- rangeland degradation with a loss of biodiversity, invasion of non-palatable species and soil erosion.

This review considers all of these land uses but focuses on the first two with salinised land expanded to include both irrigated and rain-fed environments. The landscapes of relevance are those showing ecosystem degradation but not 'terminal illness' (Kristjanson and Hobbs, 2001). They are unlikely to recover to their natural state if abandoned (Curtin, 2002) but may still be transformed into a productive and sustainable condition. This new condition may or may not be the same as the historical state.

LIVESTOCK AND LAND DEGRADATION

While grazing is often blamed for environmental degradation, there is evidence that livestock are not inherently damaging to rangelands or farming landscapes, and, in fact, may be required for their sustained health and profitability. This is not unexpected as many natural and agricultural ecosystems have evolved under grazing by herbivores. Across environments, grazing is not consistently associated with a decline in above ground net primary production or root mass (Milchunas and Lauenroth, 1993) and may have little effect on native species richness, the spread of exotic species, soil texture or soil fertility (Stohlgren et al., 1999). Livestock have been used as ecosystem engineers to improve habitat for native grassland birds (Derner et al., 2009) and livestock systems were appropriate for maintaining biodiversity integrity in South African grasslands (O'Connor and Kuyler, 2009). Exclusion from grazing in a Mediterranean environment has caused a decline in species diversity as the most competitive plants dominate (Noy Meir et al., 1989); in effect grazing reduced the advantages of competitive plants, thus allowing more species to coexist. In sub-Saharan Africa, species richness in rangelands showed the ability to recover rapidly from grazing with a balance between grazing and recovery periods the preferred option for both livestock and species diversity (Yayneshet et al., 2009a). Moderate grazing has, in some cases created highly resilient and ecologically sound systems while undergrazing has resulted in dense woody growth and reduced species diversity — a situation that is both difficult to manage and a fire hazard (Perevolotsky and Seligman, 1998). Establishing annual plant-based agriculture in fragile land systems has resulted in salinisation and soil erosion over time (Hatton and Nulsen, 1999). Similarly, conversion of rangelands into intensive crop/fodder production has also led to progressive loss of diversity, species connectivity and ability to recover (Lambin et al., 2001). There is good evidence that well-managed livestock in either a grassland, shrubland or mixed crop/livestock system offer an efficient and sustainable method of increasing the production of high quality food with minimal impact on natural resources (Tilman et al., 2002).

Overgrazing however, has been shown to reduce species diversity, genetic variation within species and to deplete seed banks of annual species (Aarssen and Turkington, 1985; Osman and Cocks, 1992). It may also reduce soil microbial biomass (Holt, 1997) and damage soil structure in both extensive rangeland (du Toit, 2005) and crop dominant farming systems (Proffitt et al., 1993). A combination of inflexibility in stocking rates and a highly variable climate have also contributed to episodic periods of intense grazing pressure and land degradation (Illius and O'Connor, 1999; O'Reagain et al., 2009).

So, an ecological and agricultural case can be established for the responsible use of livestock in degraded landscapes, but the reality is that livestock will only be grazed responsibly if the owner or user receives a benefit in the process. By providing both a profitable and sustainable option, the responsible management or reveg-etation of degraded or partly degraded landscapes may take place through the expenditure of private rather than public funding. Given the vastness of the landscapes in question and the urban priorities for the expenditure of public funds, significant progress will rely on solutions that are profitable. The alternative is to become dependent on regulation and policy. The key role of profit, rather than subsidies in the adoption of improved agricultural practices on low potential lands has been reviewed and supported by others (Barbier, 1997) and provides the basis for the strategies outlined below.

MANAGING EXPECTATIONS

Expectations for degraded landscapes need to be realistic and the development of successful grazing systems requires innovative approaches. These must address long term sustainability of soil, plant and animal production systems. The prescriptive process to feeding livestock, developed by livestock farmers and scientists for landscapes with high production potential, is inappropriate. This approach involves the application of knowledge on the nutritive and feeding value of a plant, breeding, selecting and sowing a small number of these plants with highest perceived feeding value and then managing grazing to maximise pasture growth and persistence. Such feeding systems by definition rely on minimal plant diversity. They function effectively provided high inputs are supported by high levels of production and product prices. High yields often can only be sustained with increasing levels of inputs (e.g. herbicides and fertiliser) and, the effectiveness of these inputs may decline over time. Features of plants that confer agronomic value are rarely the same as the features that confer survival value (Donald, 1963). Whether these systems become 'stressed' in the long term through continued intensification with stagnant or declining output remains to be seen (Lambin et al., 2001); what is certain is that high input, high output systems are not appropriate for degraded land or land at risk of degradation.

Degraded landscapes are often associated with spatial variation in the edaphic environment and with low and/or variable rainfall, so they are rarely able to support high levels of management or production from monocultures of highly productive plants. Attempts to establish equilibrium systems based on average rainfall and biomass production are likely to fail (Oba et al., 2001a) and may create seasonal food shortages and induce desertification. This is not surprising given the large between-year variation in biomass production in arid, semi-arid and Mediterranean-type environments (Rossiter, 1966; Ash and McIvor, 2005). Heterogeneous landscapes are more likely to support a diverse species composition that will fluctuate over time. These mixed plant assemblages are not only better suited to spatial and temporal variation, they also have a reduced risk of total failure in response to environmental or biological stressors such as drought, diseases or pest infestations.

Appropriate plants are likely to consist of a mixture of native and introduced plant species that each occupy a niche within the micro-environment and fulfil much more than the role of a feed supply. There are complex management issues underlying the productivity and stability of any mixture. Hobbs and Morton (1999) emphasised that these agro-ecosystems are likely to be dynamic and Schulte et al. (2003) suggested spatial heterogeneity could stabilise ecosystems, even though the ecosystem may oscillate at the patch scale. Furthermore, they concluded that spatial heterogeneity could be maximised by increasing the incidence of small-scale disturbances and by minimising large-scale disturbances. In a practical grazing management sense, this suggests that set-stocking of large areas will lead to reduced stability of a plant mixture.

Saline landscapes provide a practical example of spatial heterogeneity. Norman et al. (2003) identified 35 different plant species growing on two highly saline revegetated sites. The high levels of plant diversity were related to spatial variability at the site and indicated that an individual species is unlikely to dominate or thrive in all functional niches. As well as occupying heterogeneous functional niches, the mixture of plants filled a range of roles; together contributing to the prevention of wind and water erosion, provision of shelter, fuel and habitat value and more efficient water use to an extent not possible with simpler plant communities.

Plant diversity also provides an opportunity to manage temporal variability. Improved pasture plants have been selected to perform with a regular and good supply of nutrients and water and, in degraded environments, usually lack tolerance to variable rainfall and are unable to respond to unpredictable and sporadic 'out-season' climatic events. This carries two penalties; one is the exacerbated risk of land degradation and the other is lost income to the landholder. An opportunity exists to revegetate and manage degraded land with a combination of plant types to actually reduce the risk to the whole-farm enterprise and the wider landscape. Niche differentiation (i.e. the use of different resources by individual species) can occur temporally as well as spatially. The concept of degraded land being managed differently to reduce overall risk is contrary to conventional thinking that sees this land as contributing to riskiness. Whilst it is degraded the risk of further damage remains, but careful intervention can have profound effects beyond the degraded land itself. For example, Monjardino et al. (2010) found that revegetating the most marginal soil types on a typical crop-livestock farm in southern Australia with perennial forage shrubs boosted whole-farm profit disproportionately; 10% of the farm area allocated to these plant types increased whole-farm profit by 20%. Thus, providing an improved seasonal distribution of feed through better management of degrading or low-productivity land classes can actually help to reduce costs e.g. through reduced reliance on expensive feed supplements (Richards et al., 1994) and increase flexibility in animal production systems. It may even help to improve animal productivity by the strategic use of quality forage to coincide with key stages of the life cycle of animals (e.g. ovulation, parturition, lactation, or weaning; see Martin et al. (2004) and Blache et al. (2008) and, in some cases, could allow producers to consider out-of-season production to attain price premiums for animal products.

The challenge is still how to achieve a predictable and profitable return from grazing such systems while at the same time providing ecosystem services that may or may not be of financial benefit to the landholder or land manager. Management that focuses on a prescriptive approach to livestock feeding is possible but risky, but one that considers plant diversity and grazing behaviours may offer better prospects.

FUNCTIONAL LANDSCAPES

Where the aim is to simultaneously create a sustainable landscape and a profitable livestock system, a primary requirement is to understand what function plants perform in both their original and modified assembly. A consideration is that livestock systems in degraded landscapes can be managed to be both productive and sustainable, but may or may not contribute to the return of the landscape to its original state. In some cases stable vegetation that provides some of the functional benefits of the original landscape, combined with the productive benefits of a profitable livestock system may be the best option available. This then provides an opportunity to design a landscape based on a range of predetermined objectives (**Table 1**). These objectives will include both profit and ecosystem

services. For example, dryland salinity threatens significant areas of farmland in southern Australia where replacement of native perennial vegetation with annual crop and pasture species has resulted in water tables rising to the surface, bringing dissolved salts with them (Peck and Hurle, 1973). Salinisation threatens biodiversity and infrastructure on a regional scale. While revegetation with the original mixture of native perennials is an unrealistic goal, it is possible to restore the hydrological balance by strategic selection and cultivation of plants that will mimic the water use of natural ecosystems (Cocks, 2003). Plant species with deep roots and high canopy cover (green leaf area) will reduce recharge (Dunin et al., 1999; Ridley et al., 2001; White et al., 2003) while the overall composition of plant communities will influence the proportion of incident rainfall that is used, or lost through deep drainage, run-off or evaporation (Dunin et al., 1999; Ridley et al., 2001; White et al., 2003). In Western Australia, this strategy has resulted in the revegetation of 10% of the one Mha of saline land with halophytic shrubs and salt-tolerant forage. The growth and seasonal distribution of vegetation improves environmental health or 'sustainability' by reducing the recharge of saline groundwater and the run-off of both salt and sediment into waterways (Bathgate and Pannell, 2002; Turner and Ward, 2002; Peck and Hatton, 2003; Ridley et al., 2004; Norman et al., 2008a). It also provides valuable out of season feed for livestock from mixed-plant assemblies that include deep-rooted species with resilience to periods of drought and species that are able to respond quickly to rainfall by producing nutritious biomass.

In farming areas threatened by salinity but showing only early signs of degradation, planting deep-rooted perennial plants can also be used to manage the water table — these do not need to be salt tolerant but will almost certainly be forage plants rather than perennial crops (Masters et al., 2006).

Within the parts of the landscape that are already saline and those other parts at risk of salinity, there are also new and innovative farming systems under development. These involve the strategic planting of trees and/or shrubs in a wide alley configuration to provide forage and shade for livestock in the summer months while allowing inter-row cropping in winter (Lefroy and Scott, 1994). Perennial and deep-rooted woody plants contribute to the control of groundwater depth and in some cases provide biomass for power generation, activated charcoal and eucalyptus oil (Lefroy and Scott, 1994; Bell et al., 2001).

Salinity is not the only concern within the dryland cropping zones of Australia. The recent move away from livestock into more intensive cropping enterprises has produced some landscapes that display characteristics of degradation including both saline and acidic soils, wind and water erosion, loss of diversity (both natural and sown) and herbicide resistant weeds. The farming systems themselves, through a lack of enterprise diversity lack resilience. There are opportunities to redesign these systems through the use of perennial plants that are adapted for dry conditions but have the ability to respond quickly to infrequent and unpredictable heavy rainfall (Revell et al., 2008). This demonstrates a clear need for integrating evolutionary and community ecology concepts into agricultural planning and design.

Shrub systems have also been successfully established in deep sandy soils where cropping is uneconomic and traditional annual pasture plants leave the soil exposed to wind and water erosion for up to six months each year. These shrubs include *Acacia saligna*, *Atriplex spp.* and *Chamaecytisus palmensis* (Tagasaste). Tagasaste has high crude protein (>18%) and dry matter digestibility (> 65%) for at least part of the year (Borens and Poppi, 1990; Assefa, 1998), although livestock production potential is probably restricted through a high tannin and alkaloid content (Assefa et al., 2008).

While these examples focus on the specific and unique problems of degradation within Australian rain-fed farming systems,

Table 1. Objective based revegetation of degraded saline land.

Objective	Plant strategy
Lower saline water table	Deep rooted salt tolerant shrubs and other perennial plants to reduce saline water table recharge
	Ground cover to increase evapotranspiration
Reduce run-off of salt and sediment into local waterways	Deep rooted perennial shrubs and other plants to reduce water and sediment movement
	Deep rooted perennial shrubs and other plants to improve the growing environment for other plants
Improve soil properties	Revegetation to improve soil texture and microbial health
	Deep rooted perennial shrubs and ground cover to reduce soil erosion
Biodiversity and habitat	Volunteer native plants grow around shrubs.
	Potential habitat for native animals, birds and insects
Aesthetics	Improved visual amenity to address societal aspirations
Improve nutrition for grazing livestock	Salt tolerant perennials:
	Produce edible out of season biomass
	Respond to out-of-season rainfall
	Source of nitrogen, sulphur and vitamin E during the dry season
	Neutraceutical benefits such as anthelmintics, antibiotics, antimicrobials
	High salt intakes to decrease protein degradation in the rumen
	Improved growing environment to allow planting of short-season annual pasture plants
	Annual grasses and legumes:
	High nutritive value
	Increased edible biomass
	Fix nitrogen in the soil
Improve the grazing environment	Shrubs provide shade and shelter from wind and rain
Improve product quality	High salt increases efficiency of wool growth and decreases carcass fat
	Environmentally friendly products

they establish principles that are more broadly applicable. The plant assembly must have the potential to form a functional and stable community under grazing and the production potential must exceed the costs of establishment (or alternative land use).

Similar shrub or alternative forage systems have been advocated in Africa, the Middle East, Asia, North and South America (McKell, 1975; Qureshi et al., 1993; Assefa, 1998) with claims that they contribute to the alleviation of fodder shortage, soil degradation, low soil fertility and fuel wood scarcity.

These are examples of livestock-driven solutions to land degradation problems.

USING MIXED PLANT ASSEMBLIES WITH LIVESTOCK IN DEGRADED LANDSCAPES

Benefits from Plant Diversity in Grazing Systems

Developing and maintaining plant mixtures adds complexity to the feed base, and will require a change in skills for land managers. We contend that opportunities exist to capitalise on the capacity of livestock to cope with diversity of forage plants on offer. In fact, grazing herbivores seek diversity (Provenza, 1996) and, with appropriate management, can perpetuate it (Ngwa et al., 2000).

Experiments under a range of environmental conditions indicate ruminants have the ability to select foods with high digestibility and

avoid those with poor digestibility and high fibre (Duncan et al., 1994; Provenza, 1995). Crude protein content also affects diet selection although its role is less clear. Growing lambs will select from a range of different crude protein levels to obtain a mixture of feeds that meet their crude protein requirement (Kyriazakis and Oldham, 1993) but avoid excessive intakes of degradable protein (Provenza, 1995). Ruminants will also select a diet with low or manageable concentrations of anti-nutritional compounds. The ability to metabolise such compounds is often related to the intake of both metabolisable energy and other non-nutritional compounds in the diet; loss of forage diversity will be accompanied by a decline in nutritive value, palatability and metabolic variety with a consequent reduced ability to deal with toxins (Provenza et al., 2003).

These grazing behaviours are consistent with the hypothesis that sheep have the ability to select across a range of feeds to balance nutritional needs or minimise metabolic cost (Forbes and Mayes, 2002). Metabolic cost is defined as the increase in energy or other nutrients that is required to maintain health and production under sub-optimal conditions. Increases in metabolic cost may be associated with diverse stresses such as imbalanced nutrient intake or parasite infection. The concept of minimisation of metabolic cost can be taken further. For example, if methane production is an indicator of the efficiency of conversion of digestible energy to metabolisable energy, then preference may extend past digestibility to include methane production. Preference testing on forage for or against
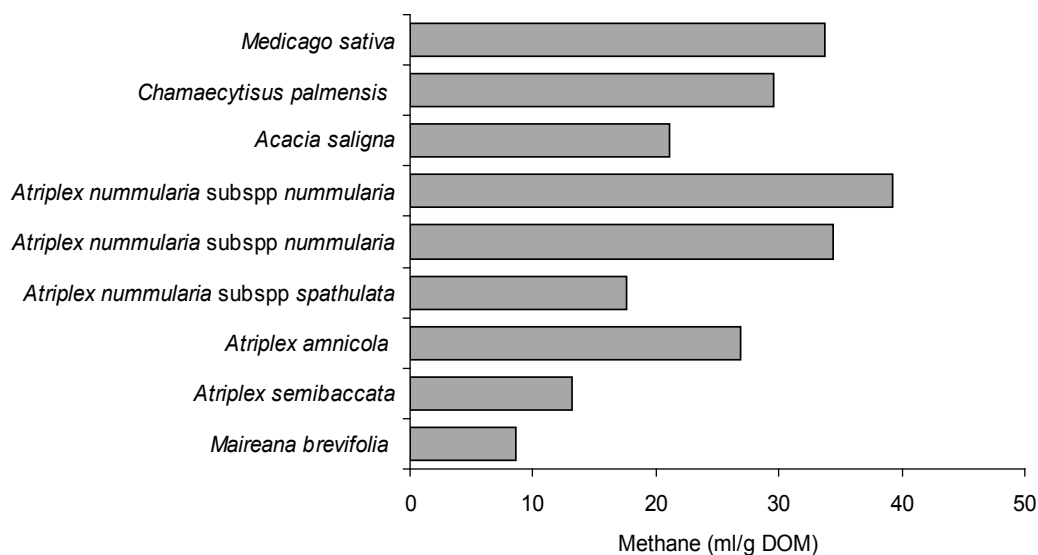
Figure 1. Methane production (ml/g DOM) measured *in vitro* (Busquet et al., 2006) for lucerne *(Medicago sativa)*, tagasaste *(Chamae-cytisus palmensis)*, orange wattle (Acacia saligna), old man saltbush *(Atriplex nummularia)* river saltbush *(Atriplex amnicola)*, creeping saltbush *(Atriplex semibaccata)* and blue bush *(Maireana brevifolia)* (Norman and Durmic, unpublished).

plants causing high methane production in the rumen is worthy of investigation. *In vitro* studies have shown wide variation in methane production from different forage sources when expressed per unit of digestible organic matter (**Figure 1**). This is consistent with observed differences in the proportion of digested energy converted to metabolisable energy across a range of plant species (Minson, 1990).

Minimisation of metabolic cost is also significant because it suggests that ruminants have the ability to optimise their diet when provided with a choice but that the choices made are not always a simple reflection of the energy and protein content of the plant options. This has been demonstrated using δ^{13}C ratios in the faeces of sheep to estimate diet selection in a mixed halophytic shrub (with a C4 photosynthetic pathway) and annual legume (with a C3 photosynthetic pathway) system. Even during times of the year when the supply of feed from annual legumes growing between shrubs was unlimited and organic matter digestibility of the pasture plants was 10% higher than in the shrubs, sheep still chose shrubs as 13% of their total feed intake (Norman et al., 2010c). The reason for the selection in this case is not obvious. Increased salt intake is likely to trigger osmoreceptors and encourage the choice of low salt alternatives, this is consistent with the reduced rate of feed intake in goats fed diets with added salt (Morand-Fehr et al., 1997). Conversely, others have shown that over the longer term, sheep will select a combination of high and low salt feed, even when the feeds are otherwise the same (Thomas et al., 2007b). Importantly, salt intake at levels above published requirements increases the flow of undegraded protein from the rumen and therefore alters the amount and relative proportions of amino acids available for absorption and also the relative proportions of absorbed energy and protein (Hemsley et al., 1975; Thomas et al., 2007a). This balance of amino acids available for absorption could therefore potentially be manipulated by the animal through the chosen level of salt intake. While these are known nutritional consequences of elevated salt intakes, selection for salt is not the only possible explanation. These shrubs are higher in fibre than spring pastures and have an unusual nitrogen, mineral, vitamin and secondary compound composition (Masters et al., 2007; Mayberry et al., 2010; Norman et

al., 2010b). These may also influence metabolic cost and subsequent selection.

A similar scenario may be expected when the forage mixture includes plants that contain tannins. Tannins at high levels will depress intake and the rate of ruminal digestion and may even cause death, but at lower levels they increase both microbial protein synthesis and amino acid flux into the small intestine (Makkar, 2003).

There are many other examples of ruminants showing preferences for dietary combinations that do not maximise the energy density of ingested feed but appear to have metabolic or health benefits that the grazing animal has the ability to recognise (Baumont et al., 2000). Rogosic et al. (2006) demonstrated that goats offered Mediterranean shrubs containing tannins and saponins consumed a variety of shrubs with different levels of the compounds in such a way as to increase shrub intake and avoid toxicosis. Goats grazing blackbrush (*Coleogyne ramosissima*), a poor quality feed that has high fibre, low crude protein and high tannins, selected older branches with lower tannins, lower energy and crude protein in preference to younger branches with higher tannin, energy and crude protein (Provenza and Malechek, 1984). Sheep grazing diverse populations of two species of saltbush were able to select and discriminate both within and between species, yet the selection could not be explained by any of the measured nutritive and anti-nutritive value characteristics of the plants (Norman et al., 2004).

This ability to utilise plants containing toxins may be facilitated through learning. Papachristou et al. (2007) demonstrated that when nutritious herbage was restricted, lambs were encouraged to learn to use different kinds of forages with a range of potentially negative plant secondary compounds. The lambs learned the benefits of mixing food with tannins, terpenes, and oxalates. Further, the lambs continued to include these compounds in their diet when they subsequently had *ad libitum* access to nutritious foods.

Meeting Nutritional Requirements

Meeting the nutritional requirements of livestock grazing in degraded environments will always be a challenge. This is primarily because

degraded landscapes produce low edible biomass. But, as discussed in the previous section, fragile or hostile environments are most likely to be converted to a functional system with a diverse plant mixture. Combining this approach with the grazing herbivore's capacity to seek nutrients and avoid toxins, there is an exciting prospect to develop sustainable grazing systems on degraded land by perpetuating diverse plant communities with managed grazing. Both overgrazing and undergrazing are likely to result in a loss of species diversity (Oba et al., 2001b) thereby reducing the ability of grazing ruminants to meet their nutritional requirements. This is perhaps even more important when considering differences between individuals in their nutrient requirements. Whilst we normally attempt to manage animals according to their average nutrient requirements, we know there are wide differences in requirements between individuals. Providing a 'cafeteria' of forages may help individuals meet their own requirements.

As livestock scientists, we have very limited knowledge of the range of nutritional and anti-nutritional compounds within many of the plants that flourish in degraded landscapes. While we may have the capacity to identify and perhaps even attempt to eradicate plants that are toxic or totally avoided by livestock, our best course of action may be to allow grazing livestock to use their sophisticated post-ingestive feedback mechanism to direct feed selection (Provenza, 1995; Weston, 2002). Within environments that are not conducive to manipulation we may need to learn from animal behaviour rather than attempt to control it.

Improving Livestock Production and Health

Complementary Feeding

Where loss of diversity is a consequence of previous overgrazing, complementary feeding can improve intake, feed conversion efficiency and therefore productive potential. Complementary feeding is defined here as a production response from two or more feeding sources that is greater than the sum of production expected when each feed source is used alone. It is a compromise between a prescriptive feeding approach, where we make all the decisions on what is best for the animal, and a system that relies on the innate ability of an animal to optimise diet selection from a range of dietary options.

The objective is to combine feed sources for improved production and can be based on cut and carry, concentrate feeds or even strategic grazing management. Examples include the provision of energy supplements to improve the utilisation of plants that contain high levels of non-protein nitrogen or to facilitate the breakdown of anti-nutritional compounds in the forage. The provision of small amounts of concentrate feeds may be all that is required. For example, growth of sheep is significantly improved when cactus cladodes, high in carbohydrate and water are fed with saltbush (*Atriplex spp*) that is high in salt, low in digestible organic matter and high in non-protein nitrogen (Ben Salem and Smith, 2008). Equally, barley grain has been shown to complement saltbush, increasing the digestibility more than would be expected from the weighted average of the two feeds (Van der Baan et al., 2004). Under grazing conditions, the provision of a barley supplement can improve live weight of sheep grazing saltbush (Franklin-McEvoy et al., 2007; Norman et al., 2008b).

Table 2. Mineral composition (on a DM basis) of 18 shrub species grown at one site Monarto, South Australia (D. Revell, J. Emms et al., unpublished data).

Species	Ca g/kg	P g/kg	Mg g/kg	Na g/kg	K g/kg	S g/kg	Cu mg/kg	Zn mg/kg
Approx. requirement[1]	1.5–4	1.3–3	1.2–1.9	0.7–1.2	5	1.5–2	5–10	20–30
Acacia saligna	6.5	0.9	3.5	4.1	15	6.3	3.3	14
Atriplex amnicola	6.8	1.9	10.5	—	28	6.1	5.0	20
Atriplex cinerea	12.4	1.7	7.0	64	33	4.7	11	19
Atriplex nummularia	14.3	1.6	7.8	73	25	7.5	8.4	42
Atriplex rhagodioides	7.1	1.9	8.2	63	26	5.9	4.8	22
Chameacytisis proliferus	6.8	1.2	4.55	1.50	8	1.2	4.0	22
Chenopodium nitrariaceum	6.1	2.4	5.6	11.9	53	4.7	3.3	24
Convolvulus remotus	5.3	1.6	3.2	1.3	16	1.6	5.3	12
Enchylaena tomentosa	3.4	1.1	1.8	—	17	2.1	8.9	15
Eremophila glabra	7.0	1.4	2.3	0.6	20	1.8	9.2	16
Eremophila longifolia	7.9	1.0	2.9	2.0	18	1.5	5.9	12
Eremophila maculata	4.3	1.4	2.4	18.8	13	1.6	4.0	14
Maireana tomentosa	3.1	2.3	2.5	—	26	3.4	8.1	15
Medicago strasseri	22.0	1.5	3.2	6.9	15	2.3	8.0	14
Rhagodia crassifolia	5.8	2.0	11.4	45	24	3.9	5.3	15
Rhagodia parabolica	5.3	1.4	6.0	30	27	3.0	4.4	16
Rhagodia preissii	7.1	1.4	8.6	31	24	3.9	13	23
Rhagodia spinescens	5.4	1.5	7.8	67	26	6.6	4.6	21

[1] Mineral requirements depend on physiological state, breed, sheep vs cattle and mineral balance. Values presented here are indicative only.

Although the focus of complementary feeding in degrading land-scapes is usually on the macro-nutrients used as energy and protein sources, there are also consequences for mineral and vitamin nutrition. This can be viewed from two perspectives. One, the traditional view, is to provide limiting minerals or vitamins in order to increase forage utilisation and increase animal performance. The other is to use forages as the source of limiting minerals and vitamins. For example some shrubs are a good source of vitamin E (Pearce et al., 2005). The shrub species listed in **Table 2** show that adequate or high levels of some minerals can be provided by certain forages. The high magnesium and sulphur in particular in some plants means that even if these shrubs provide a small proportion of total intake they have the ability to reduce the risk of grass tetany and improve the digestibility of dry forage.

Alternatively, complementary feeding can be used to dilute out limiting characteristics or other anti-nutritional compounds in one or more of the feeding alternatives. A practical example of this strategy is the feeding of cereal residues to ruminants grazing high salt shrubs. With a shrub-only diet, high salt limits feed intake and production; similarly, with a diet high in crop residue, fibre limits intake. Feed intake and production are both improved by combining the two feed sources (Le Houérou, 1992; Warren and Casson, 1992; Alicata et al., 2002). Opportunities to improve the feeding value of low digestibility grasses through the strategic use of complementary native browse species have also been described (Yayneshet et al., 2009b). Similar observations have been made when livestock have access to forages containing different toxins. For some, but not all toxins, sheep will eat more when they are offered two feeds each containing a different toxin than when they were offered a single forage with only one toxin (Burritt and Provenza, 2000). Intake almost doubled when diets containing nitrates and oxalates were fed together rather than when sheep were offered each diet separately. There was no improvement in intake when the same comparisons were made using diets containing saponin and tannin (Burritt and Provenza, 2000; Makkar, 2003).

Supplementary Feeding

The term supplementary feeding is used here to define a strategy where the nutritive value of two or more components of the diet is additive when they are fed together. Supplementary feeding is intuitively unattractive in degraded landscapes and comes with connotations of artificially elevating stocking rates well past capacity. However, while this may be the case it does not need to be so. Supplements can be used for the spatial and temporal manipulation of foraging behaviour.

Supplementary feeding to defer grazing is one example. This involves the strategic use of supplements or even full replacement rations to keep livestock off degrading land and allow either rest or forage establishment during sensitive periods. For example, in environments with regular dry and wet seasons, there is benefit in restricting grazing after initial rains. Grazing at this time may compromise seedling establishment, reduce diversity and is usually characterised by increased livestock movement and trampling damage during the search for fresh growth. The concept of deferred grazing has been studied for more than 50 years (Frandsen, 1950), but the quantification of the potential benefits for later livestock or crop production within some fragile farming systems is still not well understood (Proffitt et al., 1993).

Supplements can also be used to manipulate grazing distribution patterns. Uneven grazing is a major problem in degrading land-scapes. Management units tend to be large and there is a tendency for grazing activity to remain around an uneven distribution of water-

ing points or within an area of preferred plant species. The development of new water sources and fencing can be used to change grazing distribution but are expensive options.

Uneven grazing patterns, while initially based around forage quantity and quality, water and landscape features, also may be exacerbated by spatial memory related to previous experience and training (Bailey et al., 1996). This complicates the prediction and management of grazing distribution, but also provides an opportunity for manipulation. Bailey (2004) reported that cattle spent more time and grazed more forage in areas where molasses blocks were provided than in areas with no supplement. Supplements changed grazing patterns away from sources of water. Grazing distribution has also been manipulated through the use of free-choice mineral supplements (Ganskopp, 2001). The learning and training processes that accompany the use of such supplements means that animals may then choose to visit these new foraging areas even when supplements are no longer provided there.

The benefits of improved grazing distribution for livestock production still need to be balanced against the possible decrease in ungrazed areas and the refuge they provide for grazing sensitive plants and the consequences for native plant and animal diversity (James et al., 1999).

Strategic Revegetation

Another option is to select plants for revegetation on the basis of their ability to complement the composition and availability of feed resources already available. Where revegetation or partial revegetation is an option, there are benefits in assessing plants for palatability, nutritive value, anti-nutritional properties, shelter and possible medicinal properties as well as ecosystem benefits. These do not need to be plant breeding projects but may be localised screening programmes based on indigenous plant species with known natural advantages. Small or large scale propagation programmes are both options to support distribution requirements. A programme in Australia designed to screen and select native chenopod shrubs has recently demonstrated that there is significant variation in biomass production, palatability and nutritive value within a single species of these plants with organic matter digestibility ranging from 51% to 67% and crude protein from 12% to 19% across provenances (Norman et al., 2010a). Seeds were collected from 600 female *Atriplex nummularia* plants across southern Australia and grown together on four diverse assessment sites. This allowed prediction of heritability and indicated much of the variability in nutritive and feeding value was due to genetic rather than environmental factors. There is clearly potential to improve profitability through the selection and distribution of superior plants.

In another study, Bennell et al. (2009) screened more than 100 woody Australian plant species for nutritive value. Many were high in predicted digestibility and crude protein and have the potential to play a role in livestock-based revegetation programmes. There appears to be very few examples of plant improvement programmes of this scale that are based on the screening of native plants for livestock production. Many others have analysed and/or reviewed the nutritive value of native species, particularly shrubs (Long et al., 1999; Foster et al., 2007; Gasmi-Boubaker et al., 2008) and in some cases presented a strong case for selection or propagation of species or lines suitable for livestock production (Dynes and Schlink, 2002; Yayneshet et al., 2009a). Invariably, in these studies there is a two-fold variation in digestibility, and crude protein can vary by an order of magnitude. At the same time, the seasonal patterns of change in nutritive value can be quite different across plant species. If genetics plays a significant role in determining nutritive value in native forages

then *in situ* screening programmes, designed to identify superior plants within the relevant environment, are justified.

Complementary revegetation is not simply about the amount and nutritive value of forage produced as it will have a temporal component. Seasonal distribution has significant consequences for both the environment and animal. Seasonal variation in feed supply is a feature of many degrading environments, with periods of rapid forage growth and high feeding value followed by periods of dry, low quality feed, reduced ground cover and exposed soil. A diverse assembly of plant types that include perennial and annual species of both native and introduced plants provides a greater opportunity to develop a stable livestock system and reduce the grazing stress that often occurs during the dry season or during extended periods of low rainfall (Revell et al., 2008). Some native plants in particular have the ability to survive long periods of low rainfall and to respond quickly to rain. Providing an improved seasonal distribution of quality feed by including plants with seasonal complementarity will have both livestock and environmental benefits. While total dry matter production per year may decrease with a mixture designed to provide feed throughout the year, feed utilisation rates may well improve with animals selecting forage that would normally not be eaten, thereby increasing grazing production over the full year. From an economic point of view, the value of feed during periods of shortage far exceeds that of feed grown at other times. In the Mediterranean environment for example there is a ten-14 fold difference in marginal value of additional forage in autumn (a time of low feed quality and availability) compared with spring (high quality and availability) (Morrison and Bathgate, 1990).

Previously the combination of low edible biomass and low-moderate nutritive value of native plants, when considered as the sole source of forage, discouraged interest in their use or potential for improvement (Lefroy, 2002). Clearly a broader view that considers these plants as part of a diverse nutritional mixture with ecosystem benefits means reconsideration is necessary and may have significant benefits for agricultural systems of the future.

This is particularly the case given the challenge of using new feeding or revegetation strategies to improve livestock production and health without exacerbating landscape degradation. Landscape degradation is a possibility but not an inevitability. Many of the forage systems that are appropriate for degraded landscapes are inherently low in nutritive value (Minson and McLeod, 1970; Skarpe and Bergström, 1986; Rubanza et al., 2003). At best, the forage for much of the year is so low in digestibility that it will only support liveweight maintenance. Under such conditions there is little incentive to reduce stocking rate because nutritive value, rather than the amount of edible biomass defines the production per animal. Increasing the base nutritive value could simply be used to increase stocking rate with consequential trampling and camping damage. Alternatively, if stocking rate was maintained or even lowered, options for growth, reproduction or even lactation become realistic and greenhouse gas production per unit of production can be reduced. Greenhouse gas emissions will be lower per unit product in livestock systems with improved growth and reproduction rates. In some cases there may even be an opportunity for system change with alteration to the timing of reproduction and turnoff. Even small changes in digestibility of the edible biomass may be sufficient to encourage and support system change. Overstocking becomes a consequence of social, cultural and educational experience and not necessarily production economics.

Beyond Nutrition

The discussion to this point has focussed on improving the intake of metabolisable nutrients from forages that grow on marginal land and in minimising toxin intake or maximising the ability of the animal to detoxify anti-nutritional compounds. While this provides a broad perspective on the design and management of livestock systems in degraded landscapes, the perspective is still too narrow. There are other implications that are too frequently ignored or dismissed as too difficult. In particular, the potential neutraceutical properties of plant secondary compounds. By far the best known of these is the effect of tannins on internal parasites. Worm burden and faecal egg counts in naturally parasitised sheep are significantly reduced and growth increased by the consumption of high tannin plants (Niezen et al., 1995). In recent studies, extracts from 85 native Australian plants were screened for their ability to inhibit the development of *Haemonchus contortus* larvae *in vitro*. More than 40% of the plants showed anthelmintic properties, and tannins were the bioactive agent in only some of these plants (Kotze et al., 2009).

What is not well understood is whether parasitised sheep and cattle will actually seek out and preferentially consume plants that contain tannins or other anthelmintic compounds - are they capable of self-medication? There are many well documented examples of self-medication in non-domesticated mammals. These can be for either prevention or therapy and may play a role as stimulants, anthelmintics, laxatives, antibiotics, or as antidotes for previously consumed toxins (Lozano, 1998). Although there does not appear to be published examples of parasitised sheep or cattle selectively consuming high anthelmintic plants, there are reports that they will seek out and consume nutrients that have been depleted through parasitism such as protein (Kyriazakis et al., 1994) and sodium (Suttle et al., 1996).

Villalba and Provenza (2007) suggested that plant-derived alkaloids, terpenes, sesquiterpene lactones and phenolics may also benefit herbivores by combating internal parasites, controlling populations of fungi and bacteria, and enhancing nutrition. Vercoe et al. (2009) described the effects of 'natural bioactives' in Australian plants on acidosis and biohydrogenation *in vitro*. There are likely to be many compounds that influence immune function and the incidence of metabolic disease (Provenza and Villalba, 2010) and, while the identification and characterisation of such compounds through traditional research methods may be prohibitively expensive, the potential ability of grazing livestock to select on the basis of minimisation of metabolic cost means that over time, ingestion of compounds that reduce the need to mount a metabolically expensive immune response would be expected. This presents a logical explanation for 'self-medication'.

LIVESTOCK SELECTION AND PREPARATION

Genotype and Phenotype Selection

Just as plants species that have been bred and selected for high production systems are usually inappropriate for degraded landscapes so too are livestock. Livestock developed for high productivity systems are often not trained to cope with diversity of feed sources and not bred to thrive with between- and within-season variability.

Traditional breeds or native animals may be less selective and therefore less likely to reduce forage heterogeneity (Dumont et al., 2007) are better able to utilise rugged terrain (Bailey, 2004), have more efficient use of feed energy during periods of low feed availability or have an improved ability to metabolise and detoxify plant secondary compounds (Mead et al., 1985; Jones and Megarrity, 1986). For example, after decades of beef breeding research in Africa that

commenced in 1930, an optimum cross-breed was identified (62.5% Afrikander and 37.5% exotic Shorthorn or Hereford), which was later developed into a breed known as the Bonsmara (Cronjé, 2000).

In taking an approach to select 'appropriate animals' for the environment, it may be necessary to avoid selecting animals that partition a greater proportion of available nutrients to production only when feed supply is abundant, because these same animals are likely to be compromised when nutrient availability is limiting or variable, as is often the case in degraded landscapes (Cronjé, 2000). Consequences can include reduced reproductive and survival rates when nutrient supply is low or when other stressors occur (such as thermal stress or challenges to the immune system). This case has recently been made with high wool producing sheep in Australia. High genetic values for wool growth were accompanied by lower fat stores, lower plasma glucose and insulin and reduced reproductive turnoff — reduced fitness characteristics for survival in a harsh environment (Adams et al., 2006).

It is important to identify genotypes that are adapted to, or can cope with, the range of nutritional conditions they will be exposed to (Sinclair et al., 1998), including seasonal variation and drought events. A key point here is that where limited nutrient availability and drought events are probable, the strategy of running high stocking rates to maximise returns is risky and so less attractive. This, then, places a greater emphasis on the performance of individual animals.

While it is not always easy to identify better adapted animals within the applicable grazing environment, it may be possible to characterise local breeds for energy use and changes in body composition using ^{13}C-bicarbonate and ^{3}H-water (Makkar, 2008).

Foetal Programming

The ability of livestock to cope within a sometimes hostile environment may also be subject to gestational influence. Nutritional status at critical stages in pregnancy has the ability to have profound effects on the foetus that sometimes persist through later life. Folic acid, zinc, iron copper, vitamin A and vitamin E are of particular importance (Ashworth and Antipatis, 2001). Low birth weight will also influence glucose tolerance and response (Oliver et al., 2002). More recently there has been interest in whether exposure to nutritional or environmental stressors during gestation will result in metabolic or epigenetic changes in the offspring that will improve their ability to deal with the same stressors during later life. For example, feeding pregnant ewes salt or saltbush (*Atriplex spp*.) during part of pregnancy may lead to potentially permanent changes to the regulation of salt and water balance in the offspring (Digby et al., 2008; Chadwick et al., 2009b; Chadwick et al., 2009c). Offspring born to ewes fed saltbush during pregnancy and early lactation managed to gain body weight as weaned ten-month-old animals when they grazed saltbush for eight weeks, whereas offspring from control ewes (not fed saltbush) lost body weight (Chadwick et al., 2009a). Early life experience can also affect a ruminant's ability to deal with challenging diets. For example, lambs fed a low quality grass from one to five months of age, had a higher voluntary intake, increased nitrogen retention and higher liveweight gain when fed sorghum hay compared to conspecifics fed a higher quality diet in early life (Distel et al., 1994).

Training

While there is evidence that sheep and cattle will select diets to optimise energy, protein and toxin intake (Forbes and Mayes, 2002), such behaviour may still be affected by previous experience, at least in the short term. Small differences in grazing experience can result in large and persistent differences in grazing preference. This difference

in experience can be reflected in different feed intake and liveweight patterns in animals grazing the same area. Differences tend to be more evident in animals moving from harsh grazing environments to ones that have been improved (Arnold and Maller, 1977). Training and manipulation of experience may therefore be required under some circumstances. This may be gained from other animals (e.g. maternal influence) or may be applied externally for example, short-term elevations in stocking rate to encourage stock to try unfamiliar foods (Provenza et al., 2003) or simply through herding livestock towards or away from particular areas. Although the effectiveness of herding has been questioned, it has sometimes proved effective, particularly if combined with strategic supplement locations (Bailey, 2004).

The ability to use selection and training for the exploitation of environmental variability, rather than using technology to neutralise or avoid variability is inconsistent with modern agricultural practice, but is successfully practised in some traditional livestock systems (Krätli, 2008).

REMOTE MONITORING AND MANAGEMENT OF LIVESTOCK AND LANDSCAPE

Livestock Monitoring and Control

Strategies to improve the intake and utilisation of forages in degraded environments have the potential to induce further degradation if not accompanied by improved grazing management. Technologies are now available that allow remote monitoring of livestock condition and behaviour. This type of information provides incentive and opportunity for livestock managers to tactically manipulate feeding and production. For example, monitoring change in both livestock and forage are grazing management options. While monitoring feed supply is the more traditional method advocated in high input grazing and may also be an option for degraded environments through the use of remote sensing (Hill et al., 2004), more information and control may be available from direct livestock monitoring. Livestock will begin to lose weight, or gain weight at a reduced rate long before feed supply is exhausted, and live weight change may provide information on both available biomass and diversity of feed sources. Remote and automatic monitoring of live weight has been successfully used experimentally (Rowe et al., 2005) and can provide information on nutritional status well before it becomes clear through visual observation. Similarly, animal behaviours, such as time spent walking, grazing and preferred feeding sites are all related to feed supply and diversity. Within rangeland and Mediterranean grazing systems, walking velocity and grazing time increases and intake rate decreases when forage supply decreases (Allden and Whittaker, 1970; Bailey et al., 1996). It is now possible to monitor these behaviours remotely on at least a small number within a flock or herd (Ganskopp, 2001; Ungar et al., 2005; Thomas et al., 2008; Handcock et al., 2009). Opportunities exist to directly relate measured characteristics of animal behaviour to more specific features of ecosystem change such as the consumption of less preferred but more for vulnerable plant species or soil damage. This information on livestock condition and behaviour can then provide the basis for well informed decisions on stocking densities or relocation.

The development of virtual fencing has the potential to control the movements of sheep and cattle in extensive grazing environments. This option for remote control will complement remote animal and livestock monitoring. Although this technology is still in the very early stages of development, a prototype system has been used to successfully modify the behaviour of cattle (Bishop-Hurley et al., 2007).

Application of these techniques has the potential to optimise the combination of livestock production and ecological stability in a way that will allow the long term productive use and revegetation of degraded landscapes.

CONCLUSIONS

Degrading environments provide an opportunity for the profitable production of food from livestock. In many circumstances, a livestock system can also retrieve or sustain functionality. Whether the outcomes of profitable livestock and sustainable landscape are both achieved depends on the design and management of the system. System management within these environments requires an innovative approach integrating the skills of animal physiology and behaviour, agronomy, plant ecophysiology, soil science and ecosystem ecology and management. This integration must operate outside the narrow perspectives that often characterise these disciplines.

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Breed Diversification in South Western Uganda: Characterisation of a New Cattle Farming System

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ABSTRACT

In response to increasing land pressure due to rapidly growing population, growing demand for livestock products in urban centres and new land policies which encourage individual land ownership in Uganda, pastoralists rearing the long horned Ankole cattle in south western Uganda have now become sedentary and less dependent on communal grazing systems. Crossbreeding of Ankole cattle with the Holstein Friesian for increased milk production is taking place at a very fast rate. A new production system in which pure bred Ankole and crosses of Ankole with Holstein Friesian are reared in separate herds on one farm has now emerged in the area. As part of a programme evaluating the ecological and economic sustainability of breeding in pastoral systems, a survey of sixteen farmers selected from three sub-counties in Kiruhura District in south west Uganda was undertaken. Two sets of detailed structured questionnaires were used to collect information from the farmers. Set one was administered at the beginning of the study in April 2007, while set two was administered on a monthly basis for a period of 12 months. In addition, production data from the animals was collected monthly. Results show that crossbreeding is taking place with no defined programme, farmers still have an attachment to the Ankole cattle and that the most important challenges to the production system are insufficient pasture during the dry season and livestock diseases. The crossbreds produce significantly more milk than the Ankole and have higher live weights. There is need to formulate appropriate breeding programmes for the farmers and to develop guidelines for suitable stocking densities.

Key words: *Ankole cattle, crossbreeds, farming system, breeding programme.*

INTRODUCTION

For many years the Bahima, a pastoralist community found in south western Uganda have kept the Ankole cattle. This cattle breed is characterised by a relatively large body frame with long white horns. Their coat colour is usually solid cherry red but other colours like light brown with black stripes, red with white spots and black also exist. Ankole is a stabilised interbreed of *Bos indicus* (Zebu) and *Bos taurus* cattle (Mbuza, 1995). Traditionally the Ankole cattle play a central role in the lives of Bahima who have kept and continue to keep these animals as a source of milk for the owners, a store of wealth and pride. Typically for every 100 animals that one has, an iron bell also known as *omurebe* is tied around the favourite animal in the herd. The Ankole is adapted to the seasonal harsh climatic conditions prevalent in the south western range lands of Uganda which include low rainfall regimes leading to frequent droughts (Kisamba, 2006). In addition these animals have the ability to produce in situations where diseases and parasites are prevalent (Petersen et al., 2004; Kabi et al., 2008). It is because of these qualities that pastoralists have been able to keep this cattle breed on an extensive grazing system with minimal or no supplementation and with irregular supply of water.

According to official estimates (MAAIF, 2002) the cattle population in Uganda is around 6.1 million. Of this 50% is Long Horn Ankole, 30% the East African Short Horn Zebu, 16% Nganda, an intermediate breed of the Ankole and East African Shorthorn Zebu and the rest exotics and crosses of indigenous cattle with exotics. At a national level it is estimated that the local breeds contribute 75% of the domestic milk supply and more than 95% of all the total beef production in the country. Increasing pressure on land due to the rapidly growing population, growing demand for livestock products in urban centres and new land policies in Uganda are changing the life styles of the hitherto extensive Ankole cattle grazers. Large tracts of what used to be communal grazing land have now been fenced off and there is a shift from extensive to intensive production systems. To obtain animals with higher output, crossbreeding of Ankole with exotic breeds mainly the Holstein Friesian has begun and is taking place at a very fast rate. This has led to the emergence of a new production system where two separate herds i.e. a pure Ankole herd and a herd of Holstein Friesian–Ankole crosses are kept on one farm. In this system the Holstein Friesian-Ankole crosses are kept for commercial milk production, while the Ankole are kept for multiple reasons namely: cultural, a buffer against shock in case of prolonged drought and disease outbreak and for income through sale of live animals.

With the continuous improvement of rural infrastructure, for example through increased availability and accessibility to milk coolers and specialised milk transport vehicles to the urban centres, it is likely that cross breeding will continue at a fast rate. According to a recent report (Balikowa, 2004), south western Uganda had 61 milk collection centres with an installed capacity of 332 700 L. Since that time there has been additional investments in milk collection facilities (Wamboka, 2006) creating an even bigger demand for milk in the area. The fast rate of crossbreeding is of major concern to many and it is now believed that the Ankole breed famous for its gracefulness and long horns is now threatened with extinction (Rice, 2008).

Whereas the crossbreds produce more marketable product, they can only do so under good climatic conditions, low disease pressure

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and short drought periods. This necessitates considerable invest-ments in the control of external and internal parasites, overall disease control and in the excavation of water holding facilities. The animals need to be well managed so that there is a right balance between available pasture and the needs of the animals. The emerging pro-duction system therefore raises a number of questions. Firstly, is it economically viable? Secondly, is it sustainable in the existing environ-ment? This study was conducted in Kiruhura District in south western Uganda with the objectives of understanding the new production system, husbandry practices involved and the challenges faced by farmers.

MATERIALS AND METHODS

The Study Area

Kiruhura District falls within an area referred to as the cattle corridor that stretches from the south western part of Uganda to the North East part of the county. Kiruhura is home to the ethnic pastoralist group called Bahima. The area has two rainfall seasons with peaks from March to May and from September to November and two dry spells between June to September and from December to January (Okello et al., 2005).

Data Collection

The survey was conducted through two sets of questionnaires administered to farmers keeping two genotypes i.e. the Ankole and crosses of Ankole with Holstein Friesian in separate herds on one farm. The farmers were selected randomly from three sub-counties in Kiruhura District in south west Uganda. The first set was admin-istered at the beginning of the study in April 2007 to 16 farmers, while the second set was administered to 17 farmers on a monthly basis for a period of the 12 months. The group included the initially selected 16 farmers and an additional one. The questionnaires were designed to obtain a wide range of information which included: attitude towards crossbreeding, land size, herd size and structure, herd management, disease prevalence and production challenges. On each of the participating farms production traits such as milk yield (MY) taken once a month were recorded. A correction factor of (M x 1.65) where M is the single morning milk record was used to adjust milk yields for animals milked twice daily as observed by Erd-man et al. (1995). Other production traits collected included weight of the animal estimated by heart girth measurement (HG) and body condition score (BCS) In addition calving dates and dry off dates were recorded. Some of the information collected was supported by observations during the visits.

Data Analysis

Frequency counts and means for some of the observations were cal-culated using SAS (2002). For analysis of BCS and HG the General lin-ear model SAS (2002) was used. The model included fixed effects of genotype, season, farm, interaction of breed and farm, interaction of breed and season. Covariate (age) was used to adjust for differences in ages of the cows. For analysis of milk yields the General linear model SAS (2002) was used. The model after appropriate changes included the independent variables genotype, lactation number, and farm, breeds nested in farms, interaction of breed and lactation number, season and breed, farm and season. Covariate (days in milk) was used to adjust for stages of lactation.

RESULTS AND DISCUSSION

The Nutrition and Management of Animals

In the new farming system, grazing of the animals is usually on enclosed farms. These are enclosed either by a natural fence or barbed wire. Apart from one farmer who provided hay to his animals during the dry period, the rest of the farmers grazed their animals on natural pasture and only mineral lick was provided as a supplement.

Water was supplied to the animals once daily from valley tanks that existed on the farm. Typically the water is scooped into a drink-ing trough by a stockman. Care is taken to ensure that the two geno-types are taken to the watering points at different times.

About 50% of the farmers interviewed stated that they kept the two cattle types because the crossbreds gave them more marketable milk, while the Ankole provided security in case dis-eases or prolonged drought affected the crossbreds. Another group (19.9%) stated that they still preferred to keep Ankole cattle besides the crosses because they were hardy, while others (13.3%) stated that they kept Ankole for beef production because they were easier to sell for this purpose and the crosses for milk production. Another 13.3% stated that the crossbreds were kept for income through milk sales and Ankole were kept for cultural reasons. One farmer informed the interviewer that he started crossbreeding in 1974 and that during the civil war in Uganda in 1978/9, essential inputs like acaricides were not available; he lost his entire herd of crossbred ani-mals to tick-borne diseases. Some of his Ankole survived and he was able to re-establish his crossbreeds from this surviving group.

The majority of the interviewed farmers started crossbreed-ing between 1990 and 2000 (**Table 1**).This period coincided with the time when new land policies were introduced and with improve-ments in rural infrastructure like improved road networks and rural electrification (Sserunkuma, 1998).

The farmers interviewed all kept the two genotypes in separate herds. On some farms, grazing areas for the two genotypes were demarcated while on others the same grazing land was used but at different times. Different reasons were given for rearing the animals in separate herds: 31.25% stated that the two genotypes required different levels of management, 25% stated that this was to control breeding, 12.5% stated that the animals had different grazing behav-iour. One of the farmers informed the interviewer that the Ankole tended to graze together in one pack while the crossbreds spread out in the fields while grazing. Utilisation of the pastures was therefore different for the two genotypes. This observation is also supported by a study (Huber et al., 2008) who found that Ankole heifers tended to graze in higher densities than did the crossbred heifers as they had more animals within 5 meters around focal animals than was the case with the crossbreeds. Other reasons advanced for keeping the animals in separate herds were (i) Ankole animals scare the cross-breds, (ii) different production costs are involved for the two geno-types, and (iii) crossbreds are kept purely for milk production while the Ankole are kept for meat production in which case the Ankole could be reared at places distant from the homestead.

Table 1. Frequencies of when farmers started crossbreeding.

Period (y)	Percent
1970–1979	7.69
1980–1989	15.38
1990–2000	76.92

Only 50% of farmers indicated that they carried out pasture improvement activities such as bush clearing, removal of unwanted plants, burning, planting pasture, and a combination of two or more of the above activities.

Land Size and Animal Numbers

Most farmers interviewed owned the land on which the animals were grazed. Two farmers stated they rented additional land to ensure that they had enough pastures. Land sizes ranged between 57 ha and 750 ha. Farmers were asked to indicate how much of their land had been fenced off (all of if, part of it and/ none of it). Most (82%) indicated that only parts of the land were fenced off. All farmers were settled in one part of the farm with permanent homes. However, it was clear from the discussions that there was still some degree of movement of animals taking place by some farmers. For example, animals were shifted to distant areas during periods of prolonged drought. The selected farms had a combined total of 4 886 animals of which 56% were Holstein Friesian Ankole crosses and the rest Ankole. The combined herd sizes were between 91 and 725 animals **Table 2**. On average, herd sizes of the Ankole were smaller those of the crossbreds.

Other Farm Enterprises and Sources of Labour

Farmers indicated that in addition to keeping cattle, they kept sheep and goats and had banana plantations.

On all the farms both family and hired labour was used. The number of family members engaged in the day-to-day running of the farms was between one and four, although this number increased during school holidays when additional family members were available. The number of hired labour ranged from 1–20 depending on the number of animals owned.

Mating Systems

All farmers on the study used natural mating and each farmer owned at least one Ankole and one Holstein-Friesian bull. The maximum number of Ankole and Holstein Friesian bulls was three and four respectively. Some farmers informed the interviewer that the Holstein-Friesian bulls and some of the crossbred cows had been bought from other areas where intensive farming is well established. This included areas near to Kampala (the capital city) and Kashari (near Mbarara town). On all farms there was continuous upgrading of the crossbred animals to a higher exotic grade without a defined crossbreeding programme. This is likely to result in undesirable effects in future if management is not improved in line with changes in the genotype.

Table 2. Number of animals of the different genotypes.

Ankole (n = 16)					Crossbreds (n = 16)				
Category	Mean	Min.	Max.	S.D.	Category	Mean	Min.	Max.	S.D.
Herd	134.5	59	453	93.02	Herd	171	32	325	110.5
Bulls	1.5	1	3	0.63	Bulls	1.7	1	4	0.85
Cows	59.2	20	200	41.9	Cows	73.4	15	150	48.4
Heifers	32.8	2	80	21.93	Heifers	37.3	3	100	25.49
Steers	17.90	2	42	17.50	Steers	17	2	60	23.04
Calves	32.06	6	130	11.56	Calves	44	2	148	40.86

Table 3. Disease occurrence in the two genotypes over a 12-month period.

Ankole (n=16)		Crossbreds (n=16)	
Disease	Percent	Disease	Percent
Ephemeral fever	45.45	East Coast fever	21.8
Lumpy skin disease	27.3	Brucellosis/abortions	15.6
Internal parasites	9.09	Lumpy Skin disease	15.6
East Coast fever	9.09	Mastitis	9.4
Salmonellosis	4	Eye infections	9.4
		Ephemeral fever	6.3
		Anthrax	6.3
		Anaplasmosis	3.1
		Mange in calves	3.1
		Salmonellosis	3.1
		Internal parasites	3.1
		Dystocia	3.1

In the Ankole herd only pure breeding was practised. It was not clear from where bulls or replacement animals in this herd were obtained, although according to a similar study in the same area (Wurzinger et al., 2008) farmers are likely to replace their Ankole breeding stock from their own herd or from neighbours, friends, or relatives.

Prevalence and Control of Disease

During the monthly farm visits farmers were asked to indicate diseases that had occurred in the herd and the number of cases observed (**Table 3**). The results show that in the Ankole, ephemeral fever was most common followed by lumpy skin disease. In the crossbreds there were more disease conditions observed than in the pure bred Ankole. In this group of animals East Coast fever was the most important disease followed by brucellosis and lumpy skin disease.

All farmers had special arrangements for the control of ecto-parasites. Eighty eight percent sprayed the animals against ticks using a bucket spray while 12% of the farmers indicated that they used a plunge dip. The frequency of acaricide application varied between the farms. On 81.25% of farms all the genotypes were sprayed once a week, 12.5% indicated that the animals were sprayed twice a week while the rest indicated that they sprayed Ankole weekly and the crossbreds twice weekly. On some of the farms expensive acari-cides were used only on the crossbreds, while ordinary types were used on the Ankole. There was a wide variation in the type of aca-ricide used on different farms. However judging from the frequent use of acaricides it is likely that the success of the production system depended heavily on the availability of these chemicals. Frequency of use of drenches to control internal parasites varied widely from one to four times annually.

Production

Production and body linear traits of the adult cows are given in **Table 4**. The crossbreds had significantly (P < 0.001) higher daily milk yields and had higher bodyweights than the Ankole. The higher milk production observed in crossbreds is supported by other research findings (Rege, 1998) which showed that where management is good, there is improvement in performance with increasing *Bos taurus* genes with 50% and 75% performing better.

According to statements by the farmers total milk production/d on the farms from crossbreds ranged between 260 L and 900 L while that of the Ankole ranged between 34 L and 80 L. The average price of milk/L at the time the questionnaire was administered was approximately eight US cents. It is therefore clear that the crossbreds contributed greatly to daily house hold incomes and as such are very attractive to the farmers.

The higher body weights observed in the crossbreds indicate that this group of animals will require different stocking densities than the Ankole to obtain maximum production.

Challenges

On each of the monthly visits farmers were asked to indicate the challenges they faced on their farms since the last visit. Responses were grouped into six categories (**Table 5**).

Animal health was the single most important challenge on the farms, followed by inadequate pasture during certain periods of the year. This could be an indication that farmers were overstocking or did not have adequate arrangements to feed the animals through-out the year. This was confirmed by a study (Mulindwa et al., 2009) that simulated the long term pasture production dynamics and carrying capacity of the study area. Unstable labour was also of great concern to the farmers with frequent change of stockmen on most

Table 4. Least square means of daily milk yield, body condition score and body weight.

Daily milk yield			BCS			Body weight (cows)		
Genotype	Litres	S.E.	Genotype	Score	SE	Genotype	kg*	SE
Ankole (n = 91)	2.2[a]	0.5471	Ankole (n = 1576)	3.36	0.0283	Ankole (n = 1 622)	334.[a]	2.0167
HF50% (n = 158)	10.6[b]	0.5132	HF 50% (n = 345)	3.28	0.0915	HF 50% (n = 361)	398.2[b]	6.5442
HF > 50% (n = 625)	10.1[b]	0.1758	HF > 50% (n =1602)	3.24	0.0297	HF > 50% (n = 1 641)	395.6[b]	2.1036

HF 50% — F1 Ankole Holstein; Friesian HF > 50% — crossbreds of greater than 50% Holstein. Friesian kg* converted from heart girth measurement; SE — standard error; [a] [b] means in a column with different superscripts differ significantly (P < 0.0001), n — number of observations

Table 5. Challenges faced by farmers over a 12-month period.

Challenge	Frequency (%)
Animal health (diseases in the herd)	54.2
Animal nutrition (inadequate feed for animals)	24.1
Unstable labour	18
Unstable price of milk	1.8
Security (theft of stock)	1.2
Poor milk production	0.6

farms being noticeable. This had a negative effect on routine farm operations and much time was spent on orientation of new staff. The high turnover of staff on the farms could be due to poor remuneration and working conditions. Although unstable milk prices were not given as one of the top challenges to production, farmers complained about changes in milk prices during the different seasons. Prices were highest during the dry period when there was less milk available from the farms and then dropped during the wet seasons when supply to the collection centres exceeded capacity.

CONCLUSIONS

Cattle continue to play an important role in the lives of the Bahima by providing income from the sale of milk and a store of wealth. The changing production system is in response to the changing land policies in south western Uganda and due to demand for milk products in the urban centres. With the rapidly growing population it is likely that demand will continue to grow and crossbreeding of Ankole will continue at a very fast rate. There is therefore no guarantee that the new production system will continue unhindered. Efforts by The National Animal Genetic Resources Centre and Data Bank (NAGRC & DB) to conserve the Ankole cattle through the Ankole open nucleus breeding programme in collaboration with pastoralists should be well funded by government.

The crossbreds are very attractive to farmers in south western Uganda because of their high milk yields but there is no proper breeding programme in place to support farmers in selecting their replacement stock or guiding them on the appropriate level of crossing. Simple recording systems need to be developed and introduced to the farmers so that they have a reference point from which to make choices.

The high prevalence of diseases in cattle herds remains a very important challenge to the farmers by affecting productivity of the animals. Proper disease control strategies need to be instituted by the local authorities in the area in collaboration with the central government.

The fact that farmers are facing periods of inadequate pasture availability for their stock could be an indication that at certain times of the year these farms are over stocked. Guidelines need to be developed to enable decision-making by farmers on appropriate stocking densities and appropriate actions to reduce seasonal fluctuations in production.

The high milk yields in crossbred animals do not necessarily mean that they are profitable. An in-depth cost-benefit analysis needs to be undertaken to determine the actual costs involved in producing milk under the production system.

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