A STUDY OF THE PHENOTYPIC EXPRESSION OF THE LUSTRE GENE IN SHEEP AND A CHROMOSOMAL ANALYSIS OF THIS ABNORMALITY

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ABSTRACT

Blood and skin samples were taken from sheep with both the lustre and normal phenotype for the study of chromosomal banding patterns, follicle density, and cell type distribution. The chromosomal analysis showed no significant difference in the banding pattern, when a comparison was made between normal and Lustre animals. There were however, significant differences in the follicle density analysis. All parameters measured (primary follicle density, secondary follicle density, S/P ratio, and total follicle density) showed statistically significant differences between normal and lustre animals ($P<0.1$). There was also a significant difference in the distribution of the paracortical and orthocortical cells when comparing normal sheep to lustre. In addition to this, there was a significant difference found in proportions of the different types of cells, and the fibre area ($P<0.01$).

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CHAPTER ONE

INTRODUCTION

The wool industry is very important in New Zealand, contributing to a large proportion of the staple economic income for the country. New Zealand is the third largest wool-producing nation, behind Australia and the Soviet Union. It is therefore necessary to examine the genetic and environmental bases of any abnormal wools found, to enable their exploitation if they are useful, or their elimination if they are undesirable Some breeds of sheep have naturally lustrous wool, such as the Lincoln and Leicesters. However in these cases, the lustrous trait is not controlled by a simple dominant gene, whereas in the domestic sheep, Ovis aries lustrousness is a result of a dominant gene.

Lustrous fibres have special dying properties, and this has led to some farmers showing an interest in diversifying into animals that grow lustrous fleeces.

The lustre gene in Ovis aries (domestic sheep) has been shown to follow a dominant pattern of inheritance. Romney lustre sheep and their crosses are studied in this thesis, but other breeds of sheep, such as the Merino have also been found to have the lustre gene expressed (Short ,1958; McGuirk and Short, 1967; Warwick et al, 1960).

Lustre coats have been detected in other animals, such as the rabbit (Castle and Law, 1936) , guinea pig (Robinson, 1988), and hamster (Robinson, 1972). All of these animals show similar phenotypic differences from normal animals of the species, including decreased live weight, and decreased coat (or fleece) production.

The phenotypic differences in sheep with the lustre wool as compared to normal wool, range from a total lack of crimp, and decreased live weight, to a major decrease in wool production, and an entirely different protein

composition. In this study the phenotypic traits looked at are the cell composition, and the follicle density. The follicle density is thought to be related to the major decrease in wool production. In Fig 1.1 a lustre animal is shown next to a normal animal of the same breed at the same age for comparison.

It is known that lustrous wool contains a high proportion of high sulphur proteins, containing amino acids such as cysteine. It is also known that the cortical cell with the highest proportion of high sulphur amino acids are the paracortical cells. As a result of these two pieces of information it was suggested that lustre wool would contain mainly, or only paracortical cells.

There is evidence that the lustre wool type is due to a genetic difference, (mutation), rather than any mineral deficiency in the diet as found in some types of lustre wool (Palmer, 1949). There is also evidence that it is inherited in a simple dominant fashion (Blair, 1990; Short, 1958).

As a result of these observations three main hypotheses, (expressed as null hypotheses), were advanced and researched in this thesis:

1. That the lustre mutant phenotype in sheep is not due to any major structural change in the chromosomes.

2. That the cell composition of the fibres in the lustre mutant type sheep is no different to that in normal sheep.

3. That the follicle density in the lustre mutant sheep is no different to that in normal sheep.

Each hypothesis was studied independently, using different methods of analysis. The chromosomal analysis entailed blood sampling, chromosomal harvesting and staining; the follicle density analysis required skin sampling, thin sectioning and staining; and finally the cell composition analysis required wool sampling, sectioning and staining. After staining, each preparation was examined microscopically. (See Materials and Methods section).

Fig 1.1 Lustre and Normal Sheep

The lustre animal is shown on the right with the normal animal on the left.

In the study of chromosomal and phenotypic characteristics of the lustrous gene, a sample of five mutant animals were studied as experimentals, and compared with two normal animals as controls.

The animals available for this study were located on a farm in the Waitotara region, about 30km North of Wanganui. It appears that most, if not all, of the animals have originated from two crossbred dams, (three quarters Romney and one quarter Border Leicester), and a Border Leicester ram.

In this study G-banding techniques were applied to study the chromosomes of sheep. First a standard banded karyotype was constructed from the phenotypically normal sheep. Then each chromosome from the experimental animals was analysed in depth for any inherited structural change.

For the chromosomal part of this study an assumption was made, that any major changes in the arrangement of the DNA relating to the lustrous phenotype, may be manifest as a change in the G-banding pattern of the chromosomes of the lustrous mutant sheep. If, however, the mutation was due to a small genetic change, such as a single base pair alteration in the DNA, one would not expect to detect any change in the chromosomal banding pattern. In this case, the small change could cause a cascade of gene actions, leading to a large number of phenotypic abnormalities.

In the study of follicle density, skin sections were taken from the study animals and the number of primary and secondary follicles were determined. The S/P ratio and total number of follicles were calculated. All of these parameters were compared for normal and lustre animals.

This section of the study was important, as a statistically significant difference in follicle density could explain the lower wool production of lustre sheep as compared to normal animals of the same age.

The type and distribution of cortical cells was determined from skin sections of lustre and normal sheep, and a comparison made. Lustre wool

lacks crimp, whereas normal Romney wool is highly crimped. It was suggested that the loss of crimp could be due to a difference in the cell composition of the wool fibres of lustrous sheep.

Image Analysis involves the use of a computer to analyse various aspects of an image captured by a video camera. The system used in this thesis was a computer linked to a video camera and screen. The video camera was "looking" down the microscope. The image detected by the video camera was shown on the monitor, and analysis was done using commands fed into the attached computer. This system allowed determination of percentage of paracortical and orthocortical cells, their distribution within the fibre, total fibre area, and the ellipticity ratio. The system takes only about thirty seconds to complete all of these measurements for each fibre, which is much faster than doing all of these measurements manually. In fig 3.1 the image analysis system used can be seen.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lustre Gene

The lustre gene has been shown to be inherited in a simple dominant fashion in domestic sheep, Ovis aries (Short, 1958; Warwick et al, 1960; Blair, 1990). The lustre mutation in sheep was first observed in Australia in the 1930's and in the United States in the 1950's. Lustre type sheep have been seen in New Zealand since the early 1970's. and probably occured before this but were not reported. The lustre mutation does not show any sex-linked characteristics.

The wool of a lustrous sheep exhibits lustre, or sheen, hence the name of the mutation. Both the birth coat of the lambs, and the adult fleece are highly lustrous. There are also a number of other differences in these animals compared to normal animals. The wool tends to lack crimp, which is the wave like structure in normal wool (Chapman, Short and Hyland; 1960). The wool production is markedly decreased, with the fleece weight being only 40% to 60% of that in a normal animal. It is possible that this decrease is due to a lower follicle density. The sheep exhibiting this mutation have a tendency to show a decrease in live weight when compared to normal animals of the same age as shown by McGuirk and Short (1967). A further abnormality is that the wool felts readily, even while it is still on the animal. Felting may be due to the fact that the scales on the wool fibres of lustrous animals are not aligned symmetrically as they are in normal animals (Warwick et al, 1960). In Merino`s (Short, 1958) felting tests have been done which show that the lustre wool felts about seven times faster than does normal Merino wool. The scale structure of the lustrous wool is also thought to have an effect on the increased lustrousness of this wool.

The protein composition of lustrous wool appears to be different to that of normal sheep. It has been suggested that the mutant wool is richer in high sulphur (HS) proteins, and contains fewer high tyrosine (HT) proteins (Fraser and Rogers, 1954; Gillespie and Marshall, 1989). This is

thought to be related to the cell types present in the fibres in the mutant type of wool. Paracortical cells are known to be richer in HS proteins, such as cysteine, while only orthocortical cells contain HT proteins (Mercer, Golden and Jeffries, 1954). Gillespie and Marshall (1989) confirmed that the wool of lustre mutant sheep is richer in HS proteins and contains fewer HT proteins than normal wool by means of two-dimensional gel electrophoresis of the constituent proteins of lustre mutant and normal merino wool (Gillespie and Marshall, 1989). The amino acid cysteine is thought to be important, as it forms a disulphide bridge to stabilise the protein keratin by cross-linking the peptide chains (Mercer, 1953). This evidence leads to the suggestion that the lustre mutant type of wool contains mainly, or only, paracortical cells (Menkart and Coe, 1958; Rogers, 1959). Mutations causing a change in the protein composition of the fibre, causing changes in fibre structure and properties have also been found in naked mice, and in diseases in man such as Trichothiodystrophy.

Lustre coats have been detected in other animals, such as the rabbit (Castle and Law, 1936) , guinea pig (Robinson, 1988), and hamster (Robinson, 1972). All of these animals show similar phenotypic differences from normal animals of the species, including decreased live weight, and decreased coat (or fleece) production. In some of these animals, such as the Syrian hamster it has been found to be inherited in a semidominant fashion; while in others, such as the rabbit, it has been found to be inherited in a simple recessive manner. As mentioned before the lustre gene in Ovis aries (domestic sheep) has been shown to follow a dominant pattern of inheritance. Romney lustre sheep and their crosses are studied in this thesis, but other breeds of sheep, such as the Merino have also been found to have the lustre gene expressed (Short ,1958 ;McGuirk and Short, 1967; Warwick et al, 1960).

2.2 Wool Growth and Structure

Wool and hair fibres grow from small sac-like structures in the skin called follicles. Follicles and the glands associated with them are derived as down growths from the cellular epidermis. Wool fibres start to develop within the follicles of the foetus, or unborn lamb, at the stage of two months of development, i.e. about three months before birth.

The skin of mammals consists of two main layers: a thin outer epidermis, and a thicker inner layer, the dermis. The constantly growing and actively dividing basal layer lies between the two. It plays an important part in the development of follicles. The dermis is made up of a loose connective tissue of collagen (a protein) fibres. Wool and hair are made of the protein keratin (Bell, 1970).

There are a number of steps in the formation of wool follicles. These are summarised below (Wickham, 1975):

1. Multiplication of cells in the basal layer to form a plug and, aggregation of dermal cells below the plug.

2. Plug of basal layer cells grows down into the dermis at an acute angle to the vertical.

3. Follicle plug flattens out and a growth appears on one side of the plug. Below this outgrowth another bud appears.

4. Base of follicle plug turns in to form a dome-like structure, the papilla. The erector muscle is formed in the dermis on the same side as the glands. This extends at an angle from the lower part of the follicle up to the epidermis.

5. The hair canal begins to form in the epidermis, by keratinisation (hardening and death of cells), and the appearance of intercellular spaces. The canal runs from the neck of the follicle to the upper part of the epidermis, where it runs parallel to the skin.

6. The hair cone is formed in the lower part of the follicle from elongated, keratinised cells, which will later form the inner sheath of the wool fibre.

7. Hair cone elongates up the follicle until it reaches the base of the sebaceous gland.

8. The wool fibre, and inner sheath around it are formed by the multiplication of the epidermal cells around the papilla. The fibre is pushed

up the follicle by pressure from the dividing cells below. Its tip is hardened and keratinised and rises to the level of the sebaceous gland.

9 The tip of the fibre emerges from the inner sheath cone and enters the hair canal and the tip of the fibre comes to lie parallel with the skin surface.

10. Continued pressure from below causes the fibre to arch within the canal. This raises the epidermis into a small pimple, which can be seen with the naked eye, in a foetus at this stage of development. The pressure eventually becomes too great for the epidermis ,and it ruptures. The tip of the fibre springs out above the skin surface.

Follicles are arranged in characteristic groups. There are two types of follicle in each group; the primary and secondary follicles. The primaries are the larger, and are arranged in rows in the skin. Often there are three primary follicles to a row. The secondaries are more numerous and lie to one side of the primaries. The three primaries and associated secondaries make up the follicle group. The follicle group is the unit of wool production. Secondary follicles tend to be the smaller type and grow finer fibres than the primaries. The main difference between primary and secondary follicles is that the primaries have a sudoriferous (sweat) gland and an erector muscle associated with them, whereas the secondaries do not. Both types of follicles have associated sebaceous (wax or grease) glands, although the sebaceous gland of the secondaries is often not as well developed as that of the primaries. The primary follicles are formed first in the foetus, and the secondaries are formed later.(Ryder and Stephenson, 1968).

2.3 Fibre Structure

All fibres are made up of a cortex surrounded by a protective cuticle. A central core, or medulla, may be found in some fibres.(Wickham, 1975).

The cortex consists of long cigar-shaped cells held together by an intercellular protein. The cortical cells are responsible for the main physical properties of wool, such as crimp (Horio and Kondo, 1953; Orwin, Woods and Ranford, 1984). There are two types of cells in the cortex of the wool fibres. They have differing affinities for basic dyes. The orthocortex shows

the greatest affinity for basic dyes, and is normally found on the outside of the crimp curve; while the paracortex shows less affinity, and is normally found on the inside of the crimp curve (Horio and Kondo, 1953).

The bilateral nature of the fibre was originally thought to be associated with asymmetrical keratinisation (Mercer, 1953). The paracortex side is keratinised first and to a greater extent than the orthocortex side. Keratinisation involves the formation of disulphide bonds,by the crosslinking of the amino acid cysteine. As a consequence the paracortex tends to be richer in sulphur than the orthocortex. However, Fraser and Rogers (1954), showed the bilateral structure to be present before keratinisation of the cortex. This indicated that the bilateral nature of the cortex does not originate as a result of asymmetrical keratinisation. It is possible that the asymmetrical keratinisation occurs as a result of the bilateral nature of the cortex. The wool fibres of lustrous sheep appear to be richer in high sulphur proteins, containing amino acids such as cysteine (Fraser and Rogers, 1954; Fraser, 1964), which suggests that these fibres are mainly made up mainly, or only, of paracortical cells. This would be consistent with the lack of crimp found in the wool of these sheep.

2.4 Image Analysis

It is now about twenty years since the first appearance of papers dealing with the processing of pictorial information by computer. The growth in this field has been rapid.

Picture processing, also known as image processing or image analysis, by computer encompasses a wide variety of techniques.. Most of these have been developed in response to three major problems:

a) picture digitization and coding: conversion of pictures from continuous to discrete form (digitization); compression of the results so as to conserve storage space or channel capacity.

b) picture enhancement and restoration: improvement of degraded (blurred, noisy) pictures.

c) picture segmentation and description: conversion of pictures into simplified "maps"; measurement of properties of pictures or picture parts, classification or description of pictures in terms of parts and properties.

Computer image analysis was used to analyse a number of properties of the fibres on a microscope slide. Properties analysed include the proportion of paracortical cells, the fibre area, ellipticity ratio, minimum and maximum fibre diameters, cuticle thickness, and the distance between the centre of gravity between the para- and ortho- regions. The distance between the centre of gravities indicates whether or not the fibre has the cells arranged in a bilateral fashion .

The system that was used for image analysis in this thesis was the program Vips V4. The system was arranged so that the slide of interest was placed on a microscope stage in a normal fashion. A video camera was able to be arranged to "look" down the microscope, recording the image "seen" and sending it to a video monitor for viewing. The image was then "captured" by the computer and analysis could begin using a program specific to the requirements of this study.

Image analysis is a useful technique for quick analysis of samples, although there can be problems with the initial construction of an appropriate algorithm. The problems encountered during this study were how to get suitable threshold levels, both for the detection of fibres and for the detection of the ortho- and para- parts of the fibre. However, once the algorithm was constructed the analysis was simple and fast.

2.5 Chromosomal analysis and banding techniques

Until about twenty years ago, the mitotic chromosome was seen as a uniform object, differentiated only by the presence of the centromere, or primary constriction. In some cases secondary constrictions can also be seen. A large amount of substructure has now been revealed in chromosomes using a variety of techniques. The substructure consists of chromosome bands, which appear as transverse bands of dye of different intensities. These bands are arranged in specific patterns, characteristic for specific chromosomes in any particular species. Chromosome banding has thus

become a powerful tool for detecting minute structural changes within the chromosomes.

A large number of techniques are now available for banding chromosomes. These can be classified into six main groups.

a) Q-banding. Fixed chromosome preparations are stained in a solution of quinacrine mustard, quinacrine, Hoechst 33258 or other dyes. Observation requires a fluorescence microscope and preparations are not permanent.

b) G-banding. Chromosome preparations are incubated in saline solution, with or without prior treatment in alkali, and are then stained with Giemsa. The most widely used method of G-banding involves a mild protease treatment, usually trypsin, followed by Giemsa staining.

c) R-banding. Chromosome preparations are incubated in buffer at high temperature or at a suitable pH. They are then Giemsa stained. The pattern of banding produced is essentially the reverse of that produced by G-banding.

d) T-banding. A variant of R-banding in which bands are produced mainly at the ends of the chromosomes.

e) C-banding. Chromosome preparations are treated with moderately strong alkali, followed by warm saline treatment and Giemsa staining. Prominent bands are located at the centromeric region of the chromosome.

f) Feulgen banding. Chromosome preparations are treated in mild alkali, followed by prolonged immersion in cold saline. This is followed by the Feulgen reaction.

Each class of banding technique does not necessarily produce a unique set of chromosome bands. For example, in most mammals, G and Q banding techniques produce predominantly the same set of bands.

There are a number of theories as to the mechanism of band formation in each of the main banding techniques. For example, in G-banding, the bands formed appear to be related in some unknown way to

differences in the state of protein sulphur along the chromosome. It is also thought that the formation of the coloured compound on chromosomes stained with Giemsa, relies on the presence of DNA and it's resistance to decondensation, but not to DNA concentration. Another theory regarding the formation of G bands suggests that band formation is due to protein rearrangement, rather than the presence of DNA (Motara and Holmquist, 1987). Of course, band formation could be due to a combination of all these theories, or even an as yet unknown mechanism.

A number of species have been studied using G-banding techniques. Comparisons based on G-banding range from that of sub-species, as in the case of the Muntjacs, or barking deer of Asia (Wurster-Hill and Seidel, 1985); to comparisons of two geographically isolated populations, as in the case of the creeping vole, Microtus oregon, (Libbus and Johnson, 1988).

Karyotypes of rabbit (Yerle, et al, 1987), goat (Mensher, Bunch, and Macilius, 1989) , Atlantic eel (Sola, Camerini, and Catandalla, 1984), Muridiae in Africa (Jotterand-Bellomo, 1988), Marsupialia (Rofe and Hayman, 1985), and sheep (Bhatia and Shanker, 1989;Lord and Hill, 1991), have all been undertaken using G-banding techniques. Goat (Capra hircus), and sheep (Ovis aries) G-banded karyotypes have been used to compare the relatedness of the two species (Mensher, Bunch and Macilius, 1989). In this study it was found that the terminal light band Xq29 present in the goat was absent in the sheep. This shows how G-banding techniques can also be used in evolution studies.

The above studies have shown evidence of differences in chromosome structure by G-banding techniques. The differences found can be quite significant. For example, in the case of the creeping vole, the X chromosome was larger and had a different banding pattern in the two different populations. That is, there was intraspecies variation in chromosome length and banding pattern, as seen by G-banding, dependent on where the population was situated geographically. The comparison of different species of Muntjac (Wurster-Hill and Seidel, 1985) also showed significant differences. It was found by G-banding techniques, that the banding patterns of particular chromosomes differed between the sub-species. It was suggested as a result of this that the two differed sufficiently

to justify them being called different species rather than different sub-species.

Not only can G-banding be used to identify sub-species, and differences between geographically isolated species, but these techniques have been used to try to detect differences between clinically normal animals, and those afflicted by certain abnormalities. For example a chromosomal comparison has been made between clinically normal lambs, and those affected by ovine hereditary chondrodysplasia, otherwise known as spider syndrome. (Vanek et al, 1988). In this case no difference was detected between normal and abnormal animals.

Karyotyping and analysis by G-banding techniques has previously been done in sheep. Lord and Hill (1990) used G-banded karyotypes as a means of attempting to identify sheep carrying the Booroola fecundity gene. They observed a number of anomalies which were spread throughout most of the chromosomes, but they found that none were present in all karyotypes. This suggests that the Booroola mutation is not likely to be the result of a major chromosomal alteration.

Some of the above examples suggest that it is possible to define large chromosomal changes by the use of G-banding techniques, although smaller changes in the DNA such as point mutations will not be as readily detected. The lustre mutation in Ovis aries (Domestic sheep) causes many differences in phenotype, which suggests that the mutation could be due to a large chromosomal change, such as a large deletion. If this is the case it should be possible to detect the change by G-banding techniques.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Animals

The animals available for this study are located on a farm in the Waitotara region, about 30km North of Wanganui. It appears that most, if not all, of the animals have originated from two crossbred dams, (three quarters Romney and one quarter Border Leicester), and a Border Leicester ram. All of the parents exhibit a normal wool type. It may be of interest to note that one of the first offspring, a ram, was hypoplastic. In later years, lustrous ewes were mated to a normal ram, as well as normal ewes being mated to lustrous type rams. A population of lustrous type animals was built up in this way. In 1986 two other lustrous males were observed on another farm, near to the original farm, but geographically isolated. There was no known exchange of stock between these two farms. A third farm which was even more geographically isolated, was also observed to have a lustre type ram. Again there was no exchange of stock.

3.2 Chromosome Preparation

3.2.1 Materials

Sheep blood
Giemsa R66 stain
Sorensons buffer
Colchicine
Trypsin
Sterile heparinized vacuum tubes
Glacial acetic acid/methanol fixative
MEM-alpha medium
Phytohaemagglutinin
Foetal calf serum
Absolute ethanol
KCl

3.2.2 Method of Blood Collection, Preparation and Incubation

Blood was collected from the jugular vein of each animal into a heparin- treated vacuum tube. The heparin acted to prevent the blood from clotting. About 10 to 15 ml of blood was collected from each animal (2 control, 5 mutant). When the blood was returned to the lab, culture tubes were set up as follows:

a) 5ml MEM-alpha medium.
b) 0.2ml Phytohaemagglutinin.
c) 1ml whole blood.
d) 1ml foetal calf serum.

Each tube was mixed gently, and then incubated for 72 hours at 37°C . This allowed for the growth of the lymphocytes, the cells which contain the chromosomes in blood. Ten minutes before the 72 hours was completed, the tubes were removed from the incubator, mixed gently, and 1ml of colchicine was added. The colchicine prevented the assembly of microtubules, and thus arrested the growth of the cells in metaphase. It also acted to shorten the chromosomes. On one hand this gives less chance of overlap, but it also makes it difficult to achieve good G-bands at a later stage. The culture tubes were then returned to the incubator for a further 10 minutes.

3.2.3. Chromosome harvesting

The culture tubes were removed from the incubator, and the cells were harvested by a number of centrifuging and washing steps:

a) centrifuge, 10 min, 1000 rpm.
b) remove supernatant to 5mm above pellet.
c) rotomix, 5 sec.
d) add 5ml KCl, 37°C, mix gently.
e) incubate 10 min, 37°C water bath.
f) centrifuge, 10 min, 1000 rpm.
g) remove supernatant as before.
h) fill tube with fixative, mix gently top to bottom.

i) leave at room temperature 20 min.

j) remove supernatant.

k) half fill tube with fixative, mix gently.

l) centrifuge 10 min, 1000 rpm, remove supernatant.

m) repeat steps k) and l), 4 to 5 times, or until pellet is clean white.

n) remove as much supernatant as possible without disturbing the pellet.

o) add 2 to 3 drops of fixative to give cell suspension of light turbidity.

The lymphocytes were now ready to be dropped on to slides. The slides were acid alcohol cleaned, and then dried with a dust-free cloth. The cell suspension was dropped with a Pasteur pipette on to the slides from as high as practical to give the best spread of chromosomes. The slides were then air- dried. Block staining was then done with two slides by staining in Giemsa R66 stain for 3 to 8 minutes. They were then rinsed with Sorensen's buffer. The remaining slides were kept for G-banding.

3.2.4. G-banding

A number of methods can be used to harden the chromosomes to make them sensitive to the enzyme Trypsin for G-banding. The method followed in this study involved leaving the slides at room temperature for 7 to 12 days (usually about 10 days).

The G-banding procedure was as follows:

a) dip slides in Trypsin for anywhere between 5 sec to 1 min (usually 10 seconds was optimum).

b) immerse twice in absolute ethanol.

c) wash 30 sec or more in Sorensen's buffer.

d) stain in Giemsa R66 for 3 to 8 min.

e) rinse in Sorenson's buffer, and air dry.

The slides were checked one at a time for banding, as they were stained, to ensure that they had been in the trypsin for the correct amount of time. Too long in the trypsin causes the chromosomes to appear puffy. Too little time in the trypsin will not allow band formation at all and the chromosomes will appear block-stained. Over-staining with Giemsa can

disguise the bands whereas under-staining will stop the bands showing up correctly. Achieving the best results with G-banding is initially a matter of trial and error.

3.2.5. Analysis

Once the slides had been G-banded and dried, the slides were permanently mounted on Dupex. At this stage the slides were scanned under the microscope to find the best spreads of the chromosomes.which were then photographed. The individual chromosomes were then cut out of the photograph, and matching chromosomes paired. A karyotype of the individual sheep was constructed in this way. Karyotypes of control and mutant sheep were compared in order to detect any differences in banding patterns in individual chromosomes.

3.2.6. Fixative Preparation

The cultured lymphocytes were routinely fixed with a combination of one part glacial acetic acid, and three parts methanol. About 100ml of fixative was required for each culture tube. It was important to ensure that fresh fixative was used. This was achieved by keeping it chilled in the refrigerator.

3.2.7. Preparation of Sorenson's Buffer

To prepare Sorensen's buffer:

(a) 9.08g of potassium dihydrogen phosphate was added to 500ml of deionized distilled water, and left to dissolve overnight,and

(b) 9.47g of disodium hydrogen phosphate was added to 500ml of deionized distilled water, and left to dissolve overnight.

The next day the two solutions were mixed.

3.2.8. Preparation of Giemsa Stain

Mix 5ml of Giemsa R66 stain with 50ml of Sorensen's buffer.

3.3 Skin Sampling

3.3.1 Materials

Lignavet anaesthetic solution (local anaesthetic).
Trephine
Bouin's fluid
70% ethanol
Aureomycin antibiotic powder
clippers

3.3.2 Method of Skin Sampling

Skin samples were taken from 10 lustre and 10 normal animals. The sampling site was prepared by close clipping with electric clippers on the midside position of the sheep. This area was then anaesthetised, using Lignavet solution which takes 2 to 3 minutes to have an effect. Once the area was anaesthetised the small circle of skin to be excised was defined with the Trephine by manual rotation. Having cut through the dermis and epidermis , the sample of skin was finally removed with forceps and surgical scissors. This method was used to take two samples from each sheep, 1cm in diameter. The sample was then pressed on to a small piece of numbered cardboard and dropped into a container of Bouin's fluid fixative. After 24 hrs the Bouin's fluid was decanted off and replaced with 70% ethanol. Samples can be stored for an indefinite time in the ethanol.

The area on the midside of the sheep where the sample was taken from was treated with Aureomycin antibiotic powder.

3.3.3 Preparation of Bouin's Fluid

Bouin's fluid is prepared by mixing:

(a) 75ml saturated acetic acid
(b) 25ml 40% formalin, and
(c) 5ml acetic acid

3.3.4. Preparation of the Skin Samples

Before the skin samples could be successfully sectioned, they had to be treated in such a way that the water, making up about 80% of the tissue, was replaced by wax. This was done by dehydration using a graded series of alcohols, followed by a number of steps to replace the alcohol with chloroform, then xylene, and finally wax. The machine used to carry out this procedure was the Shandon Elliot automatic tissue processor.

The steps of the procedure were as follows:

JAR	SOLUTION	TIME
1	70% Alcohol	1 hour
2	95% Alcohol	1 hour
3	Absolute Alcohol	1 hour
4	Absolute Alcohol	1 hour
5	Absolute Alcohol	1 hour
6	Absolute Alcohol	2 hours
7	Chloroform	1 hour
8	Xylene	1 hour
9	Xylene	1 hour
10	Wax (56-58°C)	2 hours
11	Wax (56-58°C)	2 hours

The wax in the samples had to be kept hot until they were embedded in paraffin wax. Embedding involved the use of a Tissue-tech embedding consul.

Once the skin samples were embedded and the wax cooled and hardened they were able to be sectioned using a microtome. Sections were transferred on to slides which were previously coated with a thin layer of adhesive. Once the slides were dry they were placed in an oven at 60°C. At this temperature the wax melted, and upon cooling the sections were stuck to the slide.

Sections were taken at different levels throughout the tissue from the same animal for the cortical cell type analysis, as it was necessary to determine if there was a significant difference in the traits measured at different levels of the fibre. For the follicle density analysis, sections were taken from the level of the sebaceous gland.

3.4 Staining Skin Sections For Follicle Density

3.4.1 Materials

Xylene	70% alcohol
absolute alcohol	Mayers Haemalum
Scotts tap water	1% Eosin

3.4.2 Methods

Upon removal from the oven the sections were stained using the Haematoxylin and Eosin method, as follows:

1. Xylene, 5 min.
2. Xylene, 5 min.
3. absolute alcohol rinse.
4. 70% alcohol rinse.
5. tap water rinse.
6. Mayers haemalum stain, 10 min.
7. tap water rinse.
8. Scotts tap water, 2 min.
9. tap water rinse.
10. 1% Eosin, 2 min.
11. tap water rinse rapidly.
12. 70% alcohol rinse rapidly.
13. absolute alcohol rinse rapidly.
14. absolute alcohol rinse rapidly.
15. Xylene rinse.
16. Xylene rinse.
17. Mount with DPX and allow to dry.

The purpose of each step or set of steps is summarised below:

(a) Steps 3 to 5 dewax and rehydrate the samples.
(b) Step 6 Mayers Haemalum contains Haematoxylin, which stains the acidic nucleus (ie DNA and RNA).

(c) Step 8 Scotts tap water is an alkaline solution which helps the Haematoxylin to attach.
(d) Step 10 Eosin stains the cytoplasm.
(e) Steps 12 to 16 dehydrate the sample, and help to differentiate the stained areas.

The follicle density was then counted within areas of 1mm diameter, using a screen microscope, and a printed circle of known diameter on a piece of transparency.

3.5 Staining Skin Sections For Cell Type Analysis

3.5.1 Materials

Xylene	Absolute alcohol
90% alcohol	70% alcohol
Eosin-yellowish	Phloxine
Methylene Blue	Potassium carbonate
Formic acid	Hydrogen peroxide

3.5.2 Methods

Once the slides were removed from the oven, they were stained to differentiate between orthocortex and paracortex in the wool fibres, using Performic acid, Methylene blue and Eosin, as follows:

1. Deparafinize in xylene.
2. Hydrate through decreasing concentrations of ethanol to water.
3. Oxidize in a freshly prepared solution of Performic acid, 1 hr.
4. Rinse in tap water.
5. Stain in polychrome Methylene blue, 5 min.
6. Rinse in tap water.
7. Decolourize orthocortical zones of fibres with 1% HCl in 70% ethanol until this zone is clear, 2-5 min.
8. Counter stain in acidifyed Eosin-Phloxine solution, 30 sec.
9. Decolourize and dehydrate through 70%, 90%, and absolute ethanol, 1min each.
10. Clear in Xylene and mount in DPX.

Paracortex appears blue, whereas Orthocortex appears pink, and if a medulla is present it appears red.

3.5.3. Preparation of Eosin-Phloxine stain

To prepare the Eosin-phloxine stain the following are mixed:

(a) Eosin-Yellowish .	1.5g
(b) Phloxine	1.0g
(c) 70% ethanol	500ml

The pH is adjusted by the addition of concentrated HCl.

3.5.4. Preparation of Polychrome Methylene Blue Stain

To prepare the polychrome methylene blue stain the following are mixed:

(a) Methylene Blue	1.0g
(b) Potassium carbonate (K_2CO_3)	1.0g
(c) Distilled water	400ml

Dissolve ingredients and boil for 30 minutes. Cool the mixture and add 3ml of glacial acetic acid. Shake until the precipitate has dissolved, and boil gently for 5 min. Cool before use.

3.5.5. Preparation of Performic Acid

To prepare Performic acid the following are mixed:

Formic acid (98/100, w/v)	25ml
Distilled water	65ml
Hydrogen Peroxide (30% w/v)	10ml

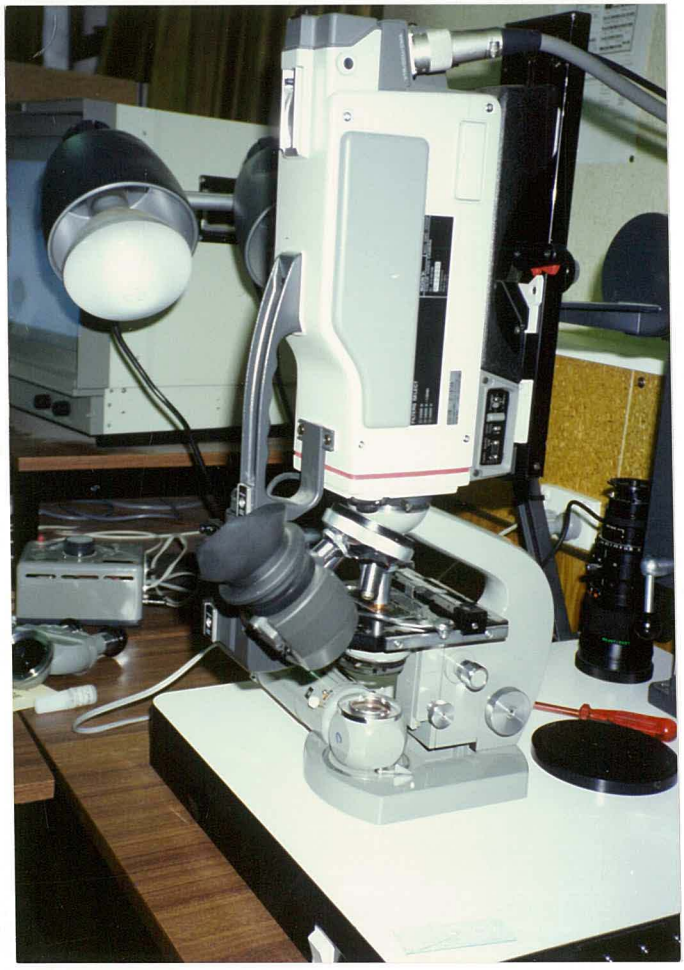
3.6. Image Analysis

Image analysis involved the use of a computer to analyse digital pictures of fibres on a microscope slide. The system used for image analysis in this thesis was the program "Vips V4." The system used was arranged so that the slide of interest was placed on a microscope stage in a normal fashion. A video camera was arranged to "look" down the microscope, recording the digital image "seen" and sending it to a video monitor for viewing. The image was then "captured" by the computer and analysis could begin using a program specific to the requirements of this study. The system used is shown in Figs 3.1 Fig 3.1(a) shows the entire system used, and (b) gives a closer look at the video camera and microscope set up. The data was analysed using minitab. The program, known as an algorithm, was set up by Dr D. Bailey of the Image Analysis Unit, Massey University.

Fig 3.1 Image Analysis System

(a) Entire system

(b) Video camera and microscope only



Before analysis could begin a calibration had to be undertaken to account for the aspect ratio, and for the area in micrometres that one pixel on the computer screen covered. To do this a circle of known diameter (92.0μ) was used, Thus when fibres were measured it was possible to convert them from pixels to micrometers. The calibration also allowed the calculation of the aspect ratio. The aspect ratio is the ratio of the width of a pixel to the length (pixels on the computer screen are rectangular). It is important for the aspect ratio to be taken into account, as results would otherwise be biased.

The program worked in the following manner: it firstly detected all complete fibres on the screen by using a threshold level of grey to distinguish the fibres from the background. After this each fibre was analysed individually and a threshold level was determined which distinguished between the orthocortical and paracortical cells. Once the two parts were detected the percentage of each was calculated and the area of the entire fibre was measured, both with the cuticle and after an estimation of it was removed. The centres of gravity were calculated for the two different parts of the fibre. The distance between them was measured as a function of the width of the fibre, along the axis on which they lay. The smaller the distance between the centres of gravity the less bilateral the distribution of the two cell types and the more concentric.

The ellipticity of the fibre was also measured. There was a small error in this measurement, due to the fact that the ellipticity measured was the width divided by length of the smallest rectangle which would fit around the fibre, rather than the smallest ellipse which would fit around the fibre. The measurement was undertaken in this way to avoid parts of the fibre extending past the edge of the ellipse, which would have resulted in an erroneous measurement. The ellipticity was measured to determine if this could have an effect on the fibre density. Increased ellipticity of fibres could cause the follicle density to decrease, thus further decreasing the wool production of the lustre sheep when compared to their normal counterparts.

To determine the error in the measurement of each parameter, one fibre was measured ten times. Each time, the slide was moved and

refocused. The mean and standard deviation was calculated and the percentage error was calculated from this. The error is shown in table 4.2. .Fibres were not detected if they touched the edge of the screen, or if they were below a specified size, this being an area lower than $200\mu^2$. The fibres were not detected if part of their outside edge was very close to the colour intensity of the background. In this case the fibre was not correctly distinguished from the background, and the computer indicated an error. These fibres were ignored.

3.7 Computer Analysis of Data

The data obtained from the Image Analysis was analysed using the statistical package, Minitab. The first step was to determine if there was a difference in the traits measured at different levels of the fibre from the same animal. To do this the means were taken from each animal at the two different levels. These were then analysed using a t-Test. None of the traits measured showed any significant difference at the different levels of the fibres ($P>0.05$). As there was no difference detected, the data from the two levels of the fibre were pooled for each animal, allowing analysis using one hundred observations rather than fifty.

Using the pooled data, means were taken for each trait for each animal; these were the values used to perform a t-Test to determine if the traits were significantly different between the Lustre and Normal animals.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Chromosomal Analysis

The chromosomal study of the lustre mutation in the domestic sheep, Ovis aries, did not yield any significant differences between the banding patterns of the normal and the lustre animals as seen in Figures 4.1 and 4.2 .

A number of changes had to be made to the original protocol for chromosome preparation, to achieve the best results. Initially the technique used was identical to that for the preparation of human chromosomes. With this technique there was very little cell growth and no chromosomes were observed in the preparation. To overcome this a change was made in both the culture medium and the type of serum used. Instead of AB serum, foetal calf serum was used. In place of Wellcome medium TC 199, MEM-alpha medium was used. These replacements allowed cell growth in the cultures. Another factor was that the blood tended to coagulate in the tubes that were used; it appeared to react with the glass tubes. The type of tube was changed from glass to plastic, thereby overcoming the problem.

Having achieved cell growth the chromosomes present would not band. In order to achieve banding, the method of hardening the chromosomes to make them susceptible to trypsin had to be changed. Initially, the slides were being heated at 60°C overnight, which did not sufficiently harden the chromosomes. This was changed to leaving them at room temperature for 7 to 12 days, with an optimum of 10 days. The temperature of the trypsin also had a marked effect on the way the chromosomes banded. It was found to work best at room temperature or below.

As is normal in studies of this type, marked differences were found between karyotypes from the same individual on the same slide, (ie treated in exactly the same way). Rofe and Hayman in 1985 also discuss differences in the banding patterns of karyotypes from the same individual . In my

study these differences were caused by the degree to which the chromosomes condensed due to the colchicine treatment, and in the intensity of staining. This differential condensation meant that in some cases bands were not able to be seen, while in other cases they were clearly visible. A standardised karyotype from the normal animals was therefore difficult to achieve. This caused difficulties in the comparisons, as chromosomes were not all at the same level of condensation and intensity of staining. For the analysis, the karyotypes with the chromosomes most intensely stained and least contracted were used. These karyotypes were the ones with the most bands clearly visible, and therefore most likely to have detectable differences. A standard karyotype was obtained and was confirmed with the chromosome banding patterns of sheep, as defined by the Committee for the Standardised Karyotype of Ovis aries, (1985).

Comparisons were carried out between normal and lustre karyotypes by comparing the banding patterns on individual chromosomes one at a time. Ten sets of good karyotypes with clear bands were compared from each animal. Any differences seen had to be observable in all of these comparisons not just in one. Any difference observed in only one set of comparisons could have been due to aberrations in staining, or it could have been due to a mutation in one individual totally unrelated to the lustre phenotype. Differences could also have been detected due to the variation in the way chromosomes at different levels of condensation stain. The comparative analysis did not yield any significant differences between the chromosomes of the normal and mutant animals. This would indicate that either there is no difference in the chromosomes of the lustre animals when compared to normal animals, or that the difference is too small to detect by analysis of banding patterns. The second is the most likely, as there must be some genomic difference in the lustre sheep when compared to the normal animals for the large amount of phenotypic difference to be detected, when it is known that this condition is inherited in a simple dominant manner in sheep. Therefore it would appear that the Lustre mutant phenotype in sheep is not due to any major structural change in the chromosomes.

4.2 Follicle Density Analysis

The section of the study dealing with the follicle density differences in the lustre and normal animals showed these differences to be significantly statistically different. The lustre animals were found to have a lower follicle density than the normal animals, which could explain why there is a lower wool production in the lustre sheep than in the normal sheep. The lower follicle density was found in all aspects of follicle density studied, ie primary follicle density, secondary follicle density, S/P ratio, and total follicle density. This agrees with the results of McGuirk and Short (1967). They also found the primary and secondary follicle densities were lower in the lustre animals. Another aspect of fleece structure which could have an effect on the wool production is the fibre diameter, which was found to be significantly lower in lustre animals compared to normal animals by McGuirk and Short (1967).

Fig 4.3 shows the staining of wool fibres for follicle density in normal animals at low and high power respectively. Fig 4.4 shows the staining of wool fibres for follicle density in lustre animals at low and high power respectively. Table 4.1 summarises the primary, secondary and total follicle densities and the S/P ratio for ten normal and ten lustre sheep.

A comparison of the lustre and normal was done by means of a t-test which gives a value indicating the probability that there is no significant difference between the means of the two groups. (The calculations of the t-Test are shown in Appendix II .) It showed that the primary follicle density and the ratio of secondary follicles to primary follicles (S/P ratio) were both marginally significantly lower in lustre sheep ($P<0.1$). The density of secondary follicles and total follicles were both significantly ($P<0.01$) lower in lustre sheep. For statistical accuracy a value of 0.1 was used as the cut off point.

fig 4.1 Chromosome Banding Pattern of Ovis aries (normal)

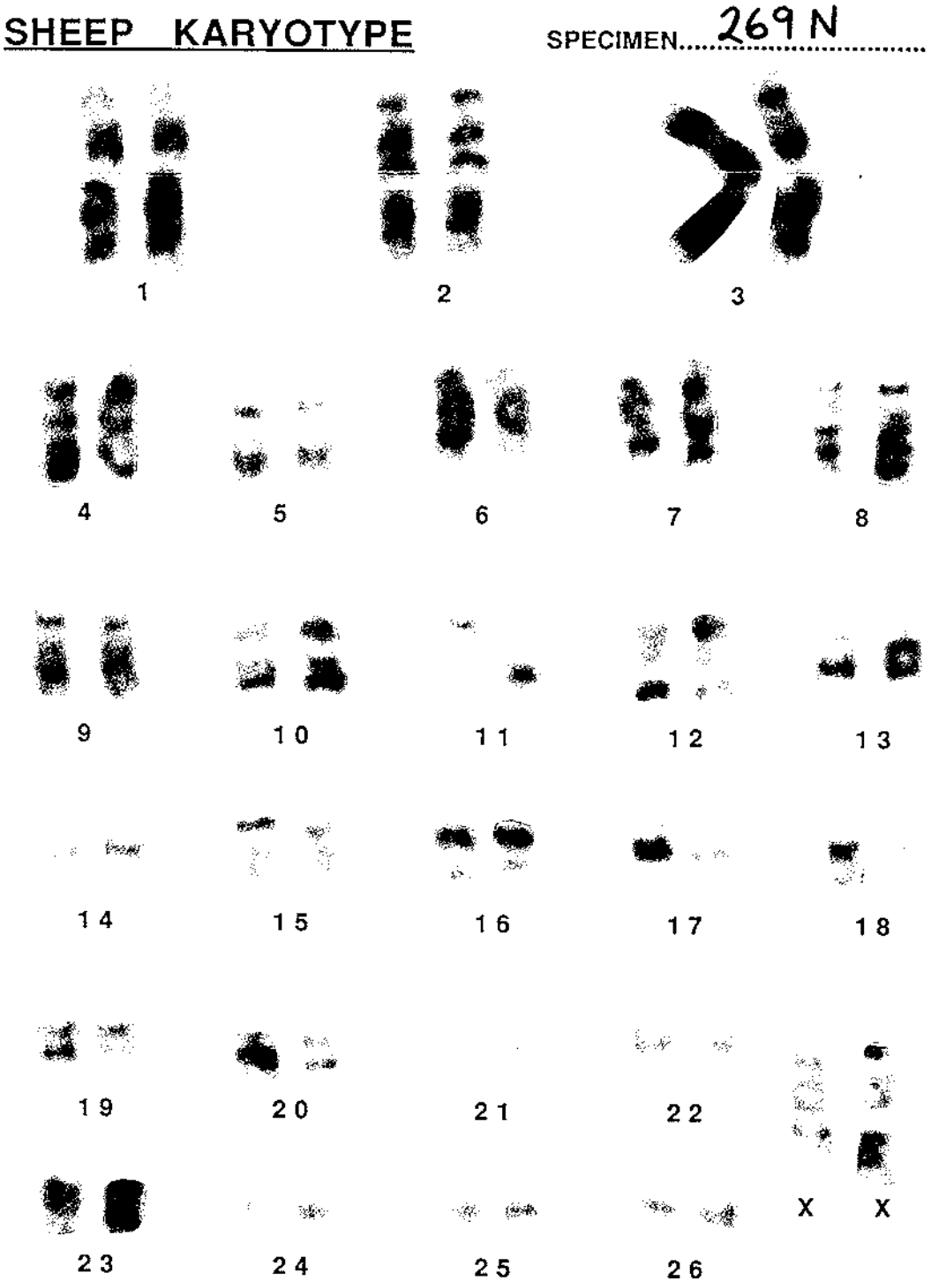


fig 4.2 Chromosome Banding Pattern of Ovis aries (Lustre)

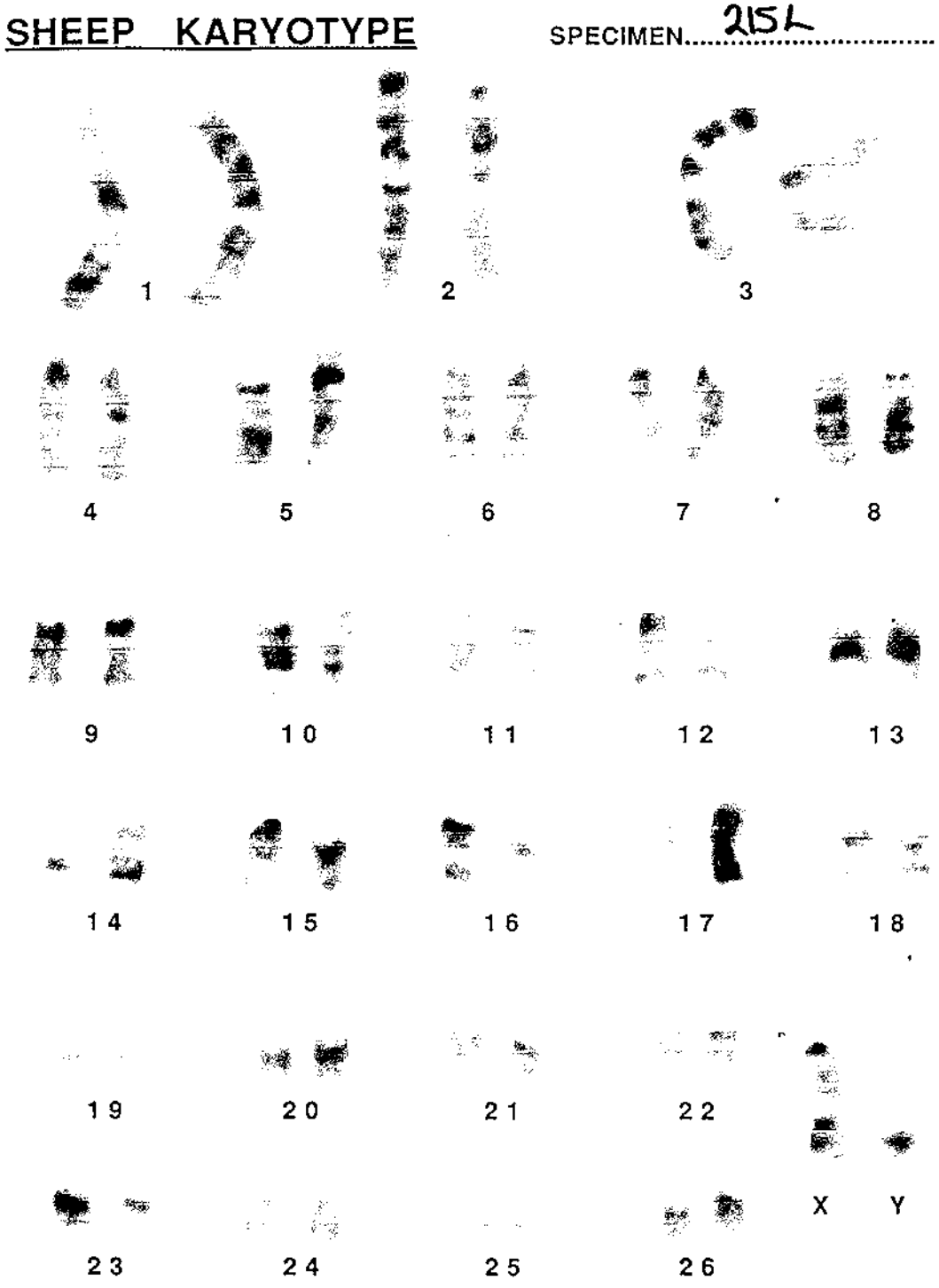
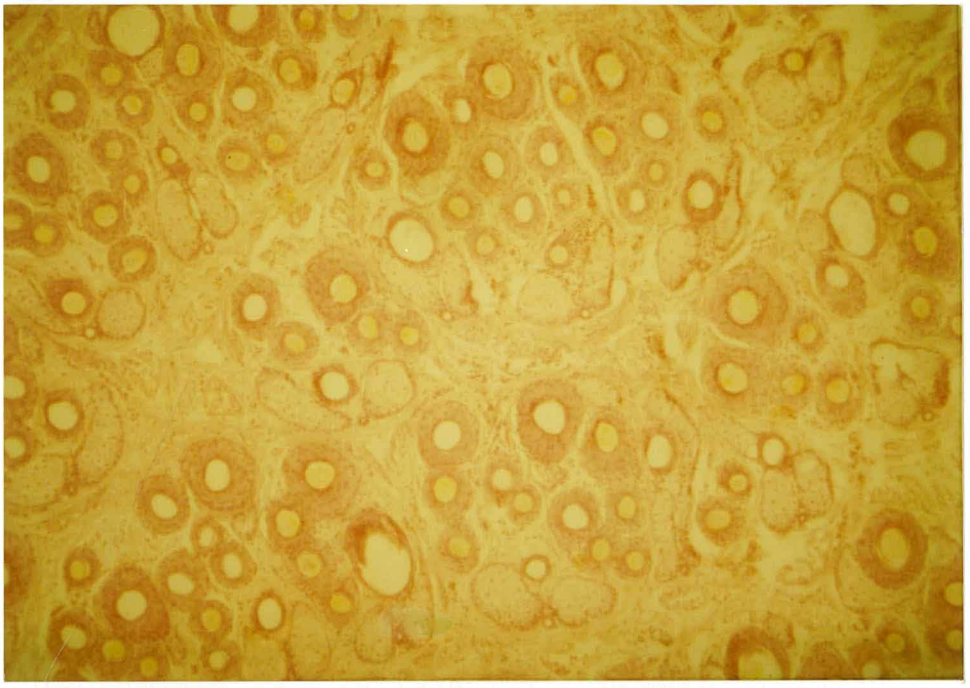


fig 4.3 Follicle Density of Wool Fibres of Ovis aries (Normal)

(a) 40 times magnification



(b) 630 times magnification

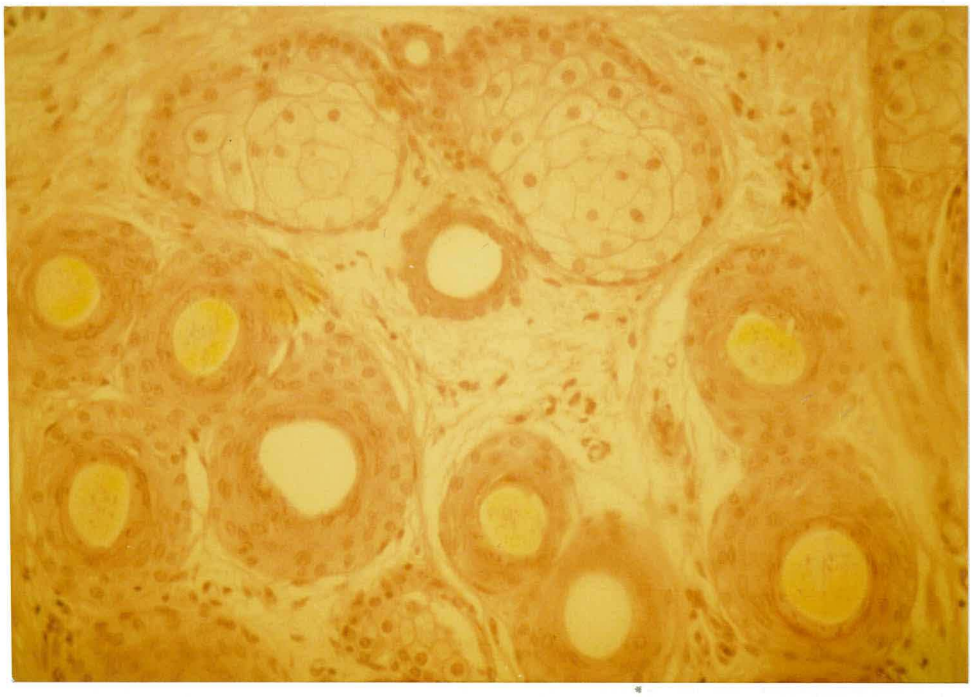
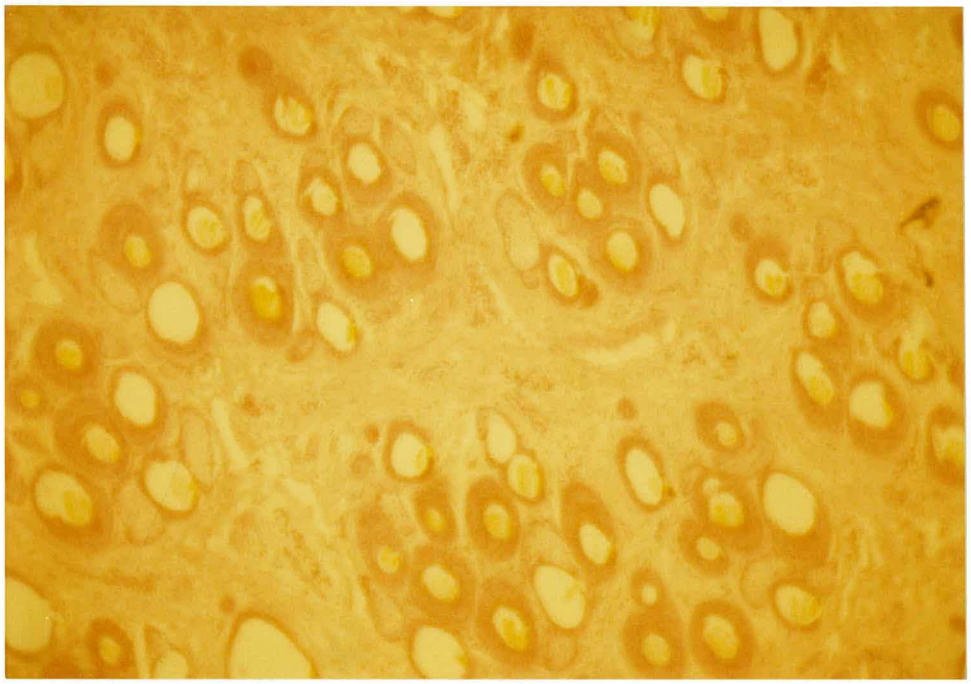


fig 4.4 Follicle Density of Wool Fibres of Ovis aries (Lustre).

(a) 40 times magnification.



(b) 630 times magnification.

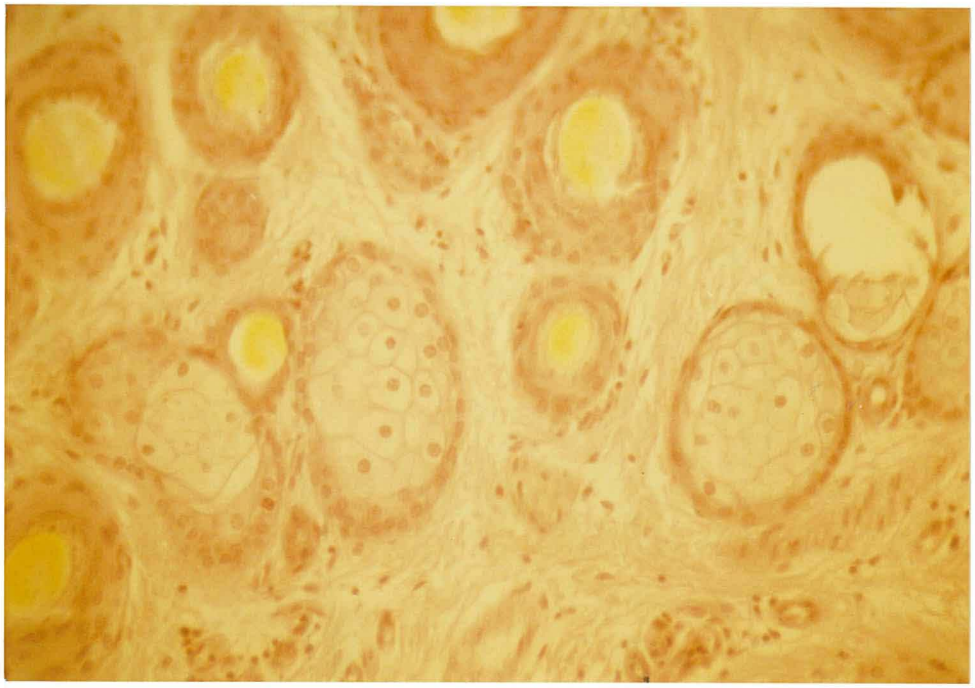


Table 4.1 Summary of Means of P, S, and Total Follicle Densities, and S/P Ratio, and Levels of Significance in Difference Between the Two Phenotypes.

Lustre					Normal			
Animal	P	S	Tot	S/P	P	S	Tot	S/P
1	6.6	25.7	32.2	4.02	8.1	38.4	46.5	4.82
2	5.3	22.7	28.0	4.51	10.3	48.2	58.5	4.9
3	7.4	27.5	34.9	3.92	5.9	41.3	47.2	7.01
4	5.5	20.3	25.8	4.41	9.0	29.1	36.6	3.93
5	6.7	32.0	38.7	5.34	6.9	40.3	47.2	6.0
6	5.2	21.0	26.2	4.43	6.1	28.9	35.0	4.93
8	8.4	36.3	44.7	4.6	7.0	38.0	45.0	5.43
9	8.7	34.4	43.1	4.21	11.6	47.8	59.4	4.19
10	7.7	32.9	40.6	4.41	9.2	38.3	47.5	4.39
Mean	6.83	28.08	34.91	4.43	8.23	38.9	46.99	5.07
Std Error	0.41	1.9	2.29	0.13	0.61	2.14	2.57	0.3
Signif.	*	**	**	*	*	**	**	*

*=P<0.1; **=P<0.01

The secondary follicle density was different to a greater degree of significance than the primary follicle density, therefore having a greater effect on the decrease in the follicle production The results shown indicate that the follicle density in lustre mutant sheep is significantly different to that of normal sheep.

This difference in follicle density is important because it is one possible reason for the lower wool production in the lustre animals when compared to the normal sheep. Staple length and fibre diameter could also have an effect. These have not been investigated in this study.

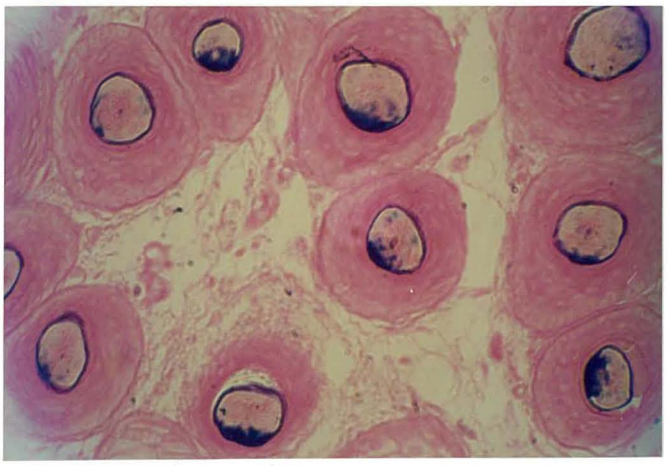
4.3 Cortical Cell Type Analysis

It is important to note that the cortical cells are responsible for the main physical properties of wool, such as crimp (Horio and Kondo, 1953; Orwin, Woods and Ranford, 1984)

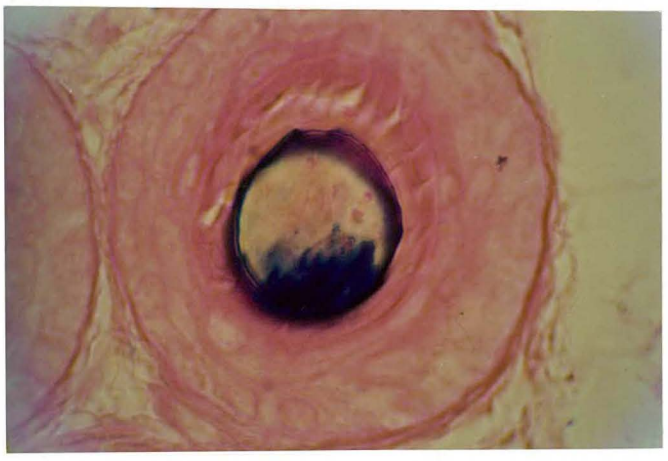
Due to the complicated staining technique that was used, the study of the cortical cell types at first proved to be difficult. The staining technique used was a slight variation on that of Clarke and Maddocks (1965). The problems lay in achieving staining with both the Eosin and the Methylene Blue. Initial trials gave staining with one but not the other. This proved to be due to the fact that the dye was not being taken up correctly, as a result of the wax remaining in the section after treatment with xylene and alcohol. To overcome this it was necessary to add another absolute alcohol step, and replace the absolute alcohol after every set of slides to ensure wax removal. When this was done good staining was achieved with the Methylene blue and good counterstaining was achieved with the Eosin-Phloxine. The orthocortical and paracortical cells were well differentiated at this stage, with the orthocortical staining pink and the paracortical staining blue.

fig 4.5 Orthocortical and Paracortical Stained Wool Fibres. Normal Animal.

(a) 40 times magnification



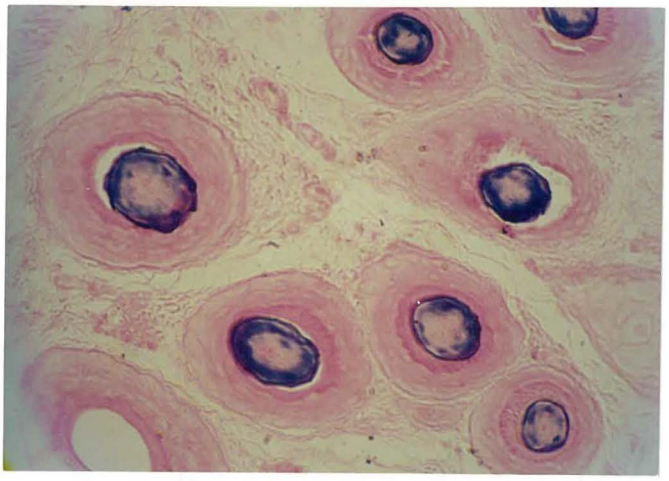
(b) 630 times magnification



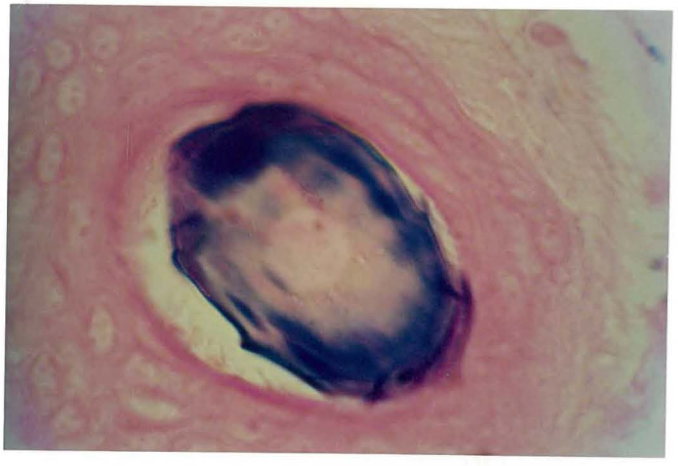
Note that there is a bilateral distribution of the two types of cortical cells. (Paracotex stains Blue and Orthocortex stains pink).

fig 4.6 Orthocortical and Paracortical Stained Wool Fibres. Lustre Animal.

(a) 40 times magnification.



(b) 630 times magnification.



Note that the paracortical cells surround the orthocortical cells

It was possible to see a qualitative difference in the distribution of the two cell types visually. In normal animals there was a bilateral distribution, whereas in the lustres the paracortex surrounded the orthocortex. Further analysis was done using Image Analysis technology to measure a number of parameters, including the area of the fibre, the proportion of paracortical cells, the ellipticity ratio of the fibre, the minimum and maximum fibre diameters, cuticle thickness, and the distance between the centre of gravity of the paracortical and orthocortical regions. There were difficulties with this due to the presence of the cuticle around the edge of the wool fibre. An algorithm had to be determined which would "ignore " the cuticle yet still count the paracortex and the orthocortex. Once this was achieved it was simply a matter of analysing the appropriate number of sections for each animal.

To determine the effectiveness of the program used for the image analysis work and any errors it may have been producing, a single fibre was measured ten times. The values for each trait measured were then analysed for mean and standard deviation and the percentage error for each was determined. The results of this can be seen in Table 4.2. The computer did not produce a percentage error for any of the traits greater than 7%. This was an acceptable level of error for the measurements.

Table 4.2 -Percentage Error of Algorithm in Measurements of Cortical Cell Type Parameters.

	%Ortho	%Para	Ellipt.	Area 1	Area 2	dCOG's
Error %	1.09	1.09	1.83	0.43	0.47	6.56

There were other minor errors in the image analysis process. The way the ellipticity was measured was not a totally accurate indication of the ellipticity of the fibre, as rather than measuring the true ellipticity, the "ellipticity" of the smallest possible rectangle to fit around the outside of the fibre was measured. This was close to the real ellipticity, but not exact.

A further minor error was the fact that the program was biased to measuring fibres of a larger area. Fibres below a certain number of pixels in area were not measured. This was in order to stop the computer from measuring small pieces of debris that were also stained, but which were generally smaller than wool fibres. However, the program was biased in this way throughout all measurement of both lustre and normal animals, so this should have had a negligible effect on the results obtained.

The results of the image analysis were processed using the statistical package, Minitab. The first analysis done using this was to compare the values for the two different sections analysed for each animal using a t-Test. When this was done it was found that there was no significant difference between the sections for each animal. This meant it was possible to pool the data from each sample for further analysis. The pooled data was used to determine whether or not there was a significant difference between the means when lustre and normal values were compared in each trait. In all cases except for the ellipticity there was a significant difference ($P<0.05$).

(a) Mean 1 and SD 1 apply to the mean and standard deviation of the top section.
(b) Mean 2 and SD 2 apply to the mean and standard deviation of the bottom section.
(c) t is the t value
(d) DF is the degrees of freedom used in the calculations
(e) P is the probability (95% certainty)
(f) Area 1 is the area of the fibre including the cuticle.
(g) Area 2 is the area of the fibre with the cuticle removed.
(h) dCOG's is the distance between the centres of gravity.

Table 4.3 shows that there is no significant difference between the measurements taken from different levels. This meant that the data from the two levels could be pooled for analysis.

The proportions of the two cell types in the lustre mutant fibres was significantly different to that in the normal wool fibres ($P<0.001$, for both paracortical and orthocortical cells). It was expected that in the lustre animals there would be mainly, or only paracortical cells present (Menkart and Coe, 1958; Rogers, 1959). It was found that this was indeed the case, with a mean of 55.72% paracortical in the mutants, and a mean of 23.07% in the normal animals. This result was also used to account for the differences in the sulphur containing amino acids present in the two types of animals. Lustre sheep have a much higher proportion of high-sulphur proteins, than do normal animals. It is known that paracortical cells have a higher sulphur content than do orthocortical cells (Fraser and Rogers, 1954; Fraser, 1964; Mercer, Golden and Jeffries, 1954) Therefore, the higher proportion of paracortical cells can explain the high sulphur content of the wool.

The area of the wool fibres was also significantly different between the two groups of animals, with the mean for the mutants being $670\mu m^2$ without cuticle, and $851\mu m^2$ with cuticle, compared to $490.7\mu m^2$ and $641.2\mu m^2$ for the normal animals. This is probably due to the fact that there is a lower follicle density in the lustre animals. To make up for this they could either produce wider or longer fibres. It appears that the fibres produced are wider, i.e. have a greater cross-sectional area. No other groups have studied the fibre cross-sectional area as in this study, although a number of investigators have measured the fibre diameter. Short (1958) showed the fibre diameter of lustrous wool fibres to be smaller than that of normal wool fibres while McGuirk and Short (1967) found no difference in the fibre diameter between the two groups of interest.

The final trait with a significant difference is the distance between the centres of gravity. This distance gives an indication of whether the cells in the fibre are arranged in a bilateral fashion, or if one cell type surrounds the other. The greater the distance between the centres of gravity, the more bilaterally segmented the fibre. In this study it was found that the lustre mutant fibres were much less bilaterally segmented than the normal ones. The mean distance between the centres of gravity was 0.1329 for the lustre

mutants and 0.2815 for the normal animals. The lustre fibres have the paracortical cells surrounding the orthocortical ones. This result could possibly account for the difference in the amount of crimping of the lustres and the normals.

Lustrous wool lacks crimp, while normal wool is highly crimped. Crimp is thought to be related to the distribution of the paracortical and orthocortical cells. Orthocortical cells tend to be found on the outside of the curve of crimp, and paracortical cells on the inside of the curve of crimp, in fibre from normal animals (Horio and Kondo, 1953; Fraser and Rogers, 1954; Kaplin and Whiteley, 1978). When the paracortical cells surround the orthocortical cells, as found in lustre mutant Romneys, the wool fibre is unable to crimp, and is held straight. Note that in lustrous Merinos in Australia there were only paracortical cells present (Short, 1958).

There was no difference in the ellipticity of the wool fibre detected when wool fibres from normal animals were compared to lustrous ones.

The analysis of the wool fibres has shown that for most traits measured there is a significant difference between lustre mutant and normal sheep wool. These differences indicate that the null hypothesis: " That the cell composition in lustre mutant sheep is no different to that in normal sheep" is in fact false. There is a statistically significant difference, as seen in Table 4.4.

Table 4.3 -Comparison of Between Level Values for Lustre and Normal Animals With Respect to Parameters of Interest.

LUSTRE				NORMAL		
TRAIT	Level 1	Level 2	t	Level 1	Level 2	t
%Ortho	44.82	42.87	0.53	70.01	83.0	-0.47
%Para	55.18	57.33	-0.58	22.11	23.98	-0.82
Ellipt.	1.511	1.323	2.07	1.262	1.336	-1.23
Area (μm^2)	830	879	-0.71	634	648	-0.36
Area(less cuticle)(μm^2)	651	654	-0.04	496	495	0.02
Centre of gravity	0.1392	0.1260	1.8	0.2923	0.2697	1.27

Table 4.4 -Comparison of the Values for Lustre and Normal Animals for Parameters of Interest.

TRAIT	MEAN(L)	MEAN(N)	t	P
%Ortho	44.28	76.93	-12.51	0.001
%Para	55.72	23.07	12.51	0.001
Ellipticity	1.382	1.301	1.47	0.16
Area (μm^2)	851	641	3.78	0.003
Area(less cuticle)(μm^2)	670	491	3.72	0.003
centre of gravity	0.1329	0.2815	-11.88	0.001

L=Lustre; N=Normal; t=t value; P=probability.

CHAPTER FIVE

CONCLUSIONS

The analysis of the chromosomes of the lustre mutant sheep, by G-banding techniques, showed no detectable differences when compared to normal animals. This result indicates that the lustre mutant phenotype in sheep is not due to any major structural change in the chromosomes.

The study of the cortical cell types showed that there were indeed differences between normal and lustre animals, with respect to a number of characteristics. Those studied and found to be significant included: the area of the fibre, the percentages of orthocortical and paracortical cells, the distribution of the two cell types i.e. the distance between the centres of gravity. The only studied characteristic which was not significantly different between the lustre and normal animals was the ellipticity of the fibre. These results indicated that the cell composition of the fibres in the lustre mutant type sheep is statistically significantly different to that in normal sheep.

The study of the follicle density also gave significant differences in all the characteristics measured, i.e primary.follicle density, secondary follicle density, total follicle density, and the S/P ratio. It was found that the lustre sheep had lower follicle density for all of these parameters than did the normal sheep. This result can explain the fact that the lustre animals have a reduced wool production compared to normal animals of the same age. The null hypothesis: "That the follicle density in the lustre mutant sheep is no different to that in normal sheep." is not correct, and it was found that there is a significant difference.

APPENDIX I

Tables of Follicle Density.

The following tables show the results of counting follicles on individual slides.

Slide 1

P	5	6	8	7	7	7	8	6	7	5	**6.6**
S	31	34	33	28	27	25	16	19	21	23	**25.7**
Tot.	35	40	41	35	34	32	24	25	28	28	**32.2**
S/P	6.2	5.67	4.13	4.0	3.86	3.57	2.0	3.17	3.0	4.6	**4.02**

Slide 2

P	5	7	6	7	6	5	3	4	5	5	**5.3**
S	28	23	31	18	24	16	21	22	16	28	**22.7**
Tot.	33	30	37	25	30	21	24	26	21	33	**28.0**
S/P	5.6	3.29	5.17	2.57	4.0	3.2	7.0	5.5	3.2	5.6	**4.51**

Slide 3

P	5	11	7	8	9	7	5	8	6	8	**7.4**
S	25	26	31	23	21	28	22	30	34	35	**27.5**
Tot.	30	37	38	31	30	35	27	38	40	43	**34.9**
S/P	5.0	2.36	4.43	2.88	2.33	4.0	4.4	3.75	5.67	4.38	**3.92**

Slide 4

P	4	6	7	5	7	3	8	5	2	8	**5.5**
S	18	22	17	25	16	20	18	24	19	24	**20.3**
Tot.	22	28	24	30	23	23	26	29	21	32	**25.8**
S/P	4.5	3.67	2.43	5.0	2.29	6.67	2.25	4.8	9.5	3.0	**4.41**

Slide 5

P	3	6	5	8	6	10	8	6	9	6	**6.7**
S	34	32	32	36	34	30	36	23	30	33	**32.0**
Tot.	37	38	37	44	40	40	44	29	39	39	**38.7**
S/P	11.3	5.33	6.4	4.5	5.67	3.0	4.5	3.83	3.33	5.5	**5.34**

Slide 6

P	7	5	6	6	7	3	3	6	5	4	**5.2**
S	24	22	24	24	18	24	18	15	18	23	**21**
Tot.	31	27	30	30	25	27	21	21	23	27	**26.2**
S/P	3.43	4.4	4.0	4.0	2.57	8.0	6.0	2.5	3.6	5.75	**4.43**

Slide 8

P	8	5	13	9	10	9	9	8	7	6	**8.4**
S	35	38	37	34	37	39	37	36	35	35	**36.3**
Tot.	43	43	50	43	47	48	46	44	42	41	**44.7**
S/P	4.38	7.6	2.85	3.78	3.7	4.3	4.11	4.5	5.0	5.82	**4.6**

Slide 9

P	11	12	11	7	6	8	10	7	9	6	**8.7**
S	29	34	35	34	34	37	36	36	35	34	**34.4**
Tot.	40	46	46	41	40	45	46	43	44	40	**43.1**
S/P	2.64	2.83	3.18	4.86	5.67	4.63	3.6	5.14	3.89	5.67	**4.21**

Slide 10

P	6	8	9	7	5	9	9	7	8	9	**7.7**
S	34	28	30	36	27	31	32	36	39	36	**32.9**
Tot.	40	36	39	43	32	40	41	43	47	45	**40.6**
S/P	5.67	3.5	3.33	5.14	5.4	3.44	3.56	5.14	4.88	4.0	**4.41**

Slide 11

P	10	9	6	8	8	9	7	8	9	7	8.1
S	42	35	36	40	36	40	41	39	39	36	38.4
Tot.	52	44	42	48	44	49	48	47	48	43	46.5
S/P	4.2	3.89	6.0	5.0	4.5	4.44	5.86	4.88	4.33	5.14	4.82

Slide 12

P	11	13	6	7	11	8	12	10	12	13	10.3
S	48	54	39	47	52	46	51	45	48	52	48.2
Tot.	59	67	45	54	63	54	63	55	60	65	58.5
S/P	4.36	4.15	6.5	6.71	4.73	5.75	4.25	4.5	4.0	4.0	4.9

Slide 13

P	5	5	5	6	7	7	5	6	7	6	5.9
S	38	43	42	40	46	41	36	44	45	38	41.3
Tot.	43	48	47	46	53	48	41	50	52	44	47.2
S/P	7.6	8.6	8.4	6.67	6.57	5.86	7.2	7.3	6.43	6.33	7.01

Slide 14

P	6	8	9	8	9	7	6	8	7	7	9
S	27	32	33	30	28	33	26	29	20	33	29.1
Tot.	33	40	42	38	37	40	32	37	27	40	36.6
S/P	4.5	4.0	3.67	3.75	3.11	4.71	44.3	3.63	2.86	4.71	3.93

Slide 15

P	7	8	7	6	9	5	6	7	7	7	6.9
S	45	46	41	45	28	37	39	43	38	41	40.3
Tot.	52	54	48	51	37	42	45	50	45	48	47.2
S/P	6.43	5.75	5.86	7.5	3.11	7.4	6.5	6.14	5.43	5.86	6.0

Slide 16

P	6	7	5	9	7	7	5	4	5	6	**6.1**
S	32	30	33	33	28	26	27	26	26	28	**28.9**
Tot.	38	37	38	42	35	33	32	30	31	34	**35.9**
S/P	5.33	4.23	6.6	3.67	4.0	3.71	5.4	6.5	5.2	4.67	**4.93**

Slide 18

P	9	7	5	9	8	6	5	7	6	8	**7.0**
S	41	38	36	39	37	36	38	38	37	40	**38.0**
Tot.	50	45	41	48	45	42	43	45	43	48	**45.0**
S/P	4.56	5.43	5.14	4.33	4.63	6.0	7.6	5.43	6.17	5.0	**5.43**

Slide 19

P	13	11	11	9	11	14	10	12	13	12	**11.6**
S	51	46	44	47	55	47	53	42	46	47	**47.8**
Tot.	64	57	55	56	66	61	63	54	59	59	**59.4**
S/P	3.92	4.18	4.0	5.22	5.0	3.36	5.3	3.5	3.54	3.9	**4.19**

Slide 20

P	11	10	10	7	9	11	7	13	7	7	**9.2**
S	36	40	42	43	30	41	37	36	40	38	**38.3**
Tot.	47	50	52	50	39	52	44	49	47	45	**47.5**
S/P	3.27	4.0	4.2	6.14	3.33	3.73	5.29	2.77	3.71	5.43	**4.39**

(a) The numbers on the far right hand side in bold print are the averages for that line for that slide.

(b) Slides 1 to 10 are samples from lustrous animals, and slides 11 to 20 are samples from normal animals.

(c) P refers to the density of primary follicles.

(d) S refers to the density of secondary follicles.

(e) Tot is the total follicle density.

(f) S/P is the secondary to primary follicle ratio.

APPENDIX II

T-Tests

For S:

μ_L =28.08 μ_N=38.9

ΣX_L^2= 7392.58 ΣX_N^2=14005.33

$(\Sigma X_L)^2$=63907.84 $(\Sigma X_N)^2$=122710.09

$(\Sigma X_L)^2/n_L$=7100.87 $(\Sigma X_N)^2/n_N$=13634.454

$\Sigma(X_L-\mu_L)^2= \Sigma X_L^2-(\Sigma X_L)^2/n_L$= 7392.58 - 7100.87 = 291.71

$\Sigma(X_N-\mu_N)^2 = \Sigma X_N^2-(\Sigma X_N)^2/n_N$ = 14005.33 - 13634.454 = 370.88

$sd = \Sigma(X_L-\mu_L)^2 + \Sigma(X_N-\mu_N)^2/(n_L-1)+(n_N-1)$= 291.71 + 370.81 / 8+8 = 41.41

standard error = $\sqrt{sd^2/n_L + sd^2/n_N}$ = 41.41/9 + 41.41/9 = 3.03

t = $\mu_L-\mu_N$/ standard error

=28.09 - 38.9/ 3.03

= -3.57

Using 16 degrees of freedom, the value of t lies between 0.001 and 0.01. This indicates that there is a significant difference between the means of normal and lustre animals with respect to secondary follicle density. ($P<0.01$)

For P:

μ_L= 6.83 μ_N= 8.23

ΣX_L^2= 434.13 ΣX_N^2= 640.53

$(\Sigma X_L)^2$= 3782.25 $(\Sigma X_N)^2$= 5490.81

$(\Sigma X_L)^2/n_L$= 420.25 $(\Sigma X_N)^2/n_N$= 610.09

$\Sigma(X_L-\mu_L)^2= \Sigma X_L^2-(\Sigma X_L)/n_L$= 434.13 - 420.25 = 13.88

$\Sigma(X_N-\mu_N)^2= \Sigma X_N^2-(\Sigma X_N)/n_N$= 640.53 - 610.09 = 30.44

$sd^2= \Sigma(X_L-\mu_L)^2 + \Sigma(X_N-\mu_N)^2/(n_L-1)+(n_N-1)$ = 13.88 + 30.44/ 8+8

=2.77

standard error = $\sqrt{sd^2/n_L + sd^2/n_N} = \div 2.77/9 + \div 2.77/9 = 0.785$

$t = \mu_L - \mu_N$/standard error
= 6.83 - 8.23/ 0.785
= -1.78

Using 16 degrees of freedom the value for t lies between 0.05 and 0.1. This indicates that there is a significant difference between the means with respect to primary follicle density. (P<0.1)

For S/P:

$\mu_L = 4.43$	$\mu_N = 5.067$
$\Sigma X_L^2 = 177.79$	$\Sigma X_N^2 = 238.45$
$(\Sigma X_L)^2 = 1588.02$	$(\Sigma X_N)^2 = 2079.36$
$(\Sigma X_L)^2/n_L = 176.45$	$(\Sigma X_N)^2/n_N = 231.04$

$\Sigma(X_L-\mu_L)^2 = \Sigma X^2-(\Sigma X_L)^2/n_L = 177.79-176.45 = 1.34$
$\Sigma(X_N-\mu_N)^2 = \Sigma X^2-(\Sigma X_N)^2/n_N = 238.45-231.04 = 7.41$

$sd^2 = \Sigma(X_L-\mu_L)^2 + \Sigma(X_N-\mu_N)^2/(n_L-1) + (n_N-1) = 1.34 + 7.41/ 8+8 = 0.547$

standard error = $\sqrt{sd^2/n_L + sd^2/n_N} = \div 0.547/9+0.547/9 = 0.349$

$t = \mu_L - \mu_N$/standard error
= 4.43-5.067/0.349
= -1.83

Using 16 degrees of freedom the value for t lies between 0.05 and 0.1. This indicates a significant difference between the means with respect to Secondary to Primary ratio. (P<0.1)

For Tot:

$\mu_L = 34.91$	$\mu_N = 46.99$
$\Sigma X_L^2 = 11392.68$	$\Sigma X_N^2 = 20414.35$

$(\Sigma X_L)^2 = 98721.64$ $(\Sigma X_N)^2 = 178844.41$

$(\Sigma X_L)^2/n_L = 10969.07$ $(\Sigma X_N)^2/n_N = 19871.60$

$\Sigma(X_L-\mu_L)^2 = \Sigma X_L^2-(\Sigma X_L)^2/n_L = 11392.68-10969.07 = 423.61$

$\Sigma(X_N-\mu_N)^2 = \Sigma X_N^2-(\Sigma X_N)^2/n_N = 20414.35-19871.60 = 542.75$

$sd^2 = \Sigma(X_L-\mu_L)^2 + \Sigma(X_N-\mu_N)^2/(n_L-1) + (n_N-1) = 423.61+542.75/8+8$
$= 60.4$

Standard error $= \sqrt{sd^2/n_L + sd^2/n_N} = \div 60.4/9+60.4/9$
$= 3.66$

$t = \mu_L-\mu_N/\text{standard error}$
$= 34.91-46.99/3.66$
$= -3.30$

Using 16 degrees of freedom the value of t lies between 0.01 and 0.001. This indicates a significant difference between the means with respect to total follicle density. ($P<0.01$)

APPENDIX III

GLOSSARY

Aspect ratio: The ratio of the length to width of a pixel on a computer screen. This ratio has to be calibrated, as pixels are not square, but rectangular.

Aureomycin: Trade name for chlorotetracycline hydrochloride, an antibiotic metabolic product of Streptomyces aureofaciens, with low toxicity, effective against many viruses and bacteria.

Banding pattern: The pattern of light and dark bands observed on chromosomes by light microscopy, after treatment with trypsin and staining with Giemsa.

Bilateral distribution: of cortical cells in wool fibres. The two types of cells are found in different halves of the fibre.

Bouin's fluid: A fixative used for the storage of tissue such as skin.

Cell composition: The composition of the wool fibres with respect to the proportion of para- and ortho-cortical cells.

Centromere: The primary constriction on the chromosome, it is an indentation on the chromosome which may occur at any point along the chromosome. The position of the centromere is specific for each chromosome and is used to help identify individual chromosomes of specific species.

Chromosome: Rod-like or thread-like structures of DNA occurring in pairs in the nucleus of plant or animal cells.

Colchicine: Chemical formula $C_{22}H_{25}O_6$. An alkaloid from the root of autumn crocus, Colchinum autumnale. Used in the treatment of lymphocytes to prevent microtubule assembly, thus arresting the growth of the cells in metaphase. Acts to condense the chromosomes.

Concentric distribution: of cortical cells in wool fibres. One cell type surrounds the other, rather than being bilaterally distributed.

Crimp: The wave pattern of wool fibres in sheep.

Dominant allele: Of a pair of allelomorphic characters, the one which will be manifested if both forms are present.

Ellipticity: of wool fibres is the ratio of the length to the width of the fibre.

Eosin: Chemical formula $C_{20}H_6Br_4O_5K_2$, the potassium salt of tetrabromo-fluorescein, a red dye.

Felting: The matting together of wool fibres.

Follicle: Small sac-like cavity containing the hair root. There are primary and secondary follicles.

Follicle density: number of follicles per unit area.

Genes: Units arranged in a linear fashion on chromosomes, each having a specific effect on the phenotype.

Giemsa R66: A mixture of Methylene blue and Eosin, used particularly for staining micro-organisms, blood films and tissue culture cells.

Haematoxylin: A colouring matter extracted from dogwood, used to stain microscopic preparations.

Heparin: complex organic acid containing glucosamine, glucuronic acid and sulphuric acid. It delays coagulation of blood and is used by intravenous injection in medicine and surgery, being usually obtained from lung or liver of mammals.

High sulphur proteins: Proteins which have a high sulphur content, due to amino acids containing sulphur. e.g. cysteine.

High tyrosine proteins: Proteins which contain a lot of the amino acid tyrosine.

Hoechst 33258: A fluorescent dye which is used for staining chromosomes.

Hypoplasia: Underdevelopment; deficiency. (adj. hypoplastic). e.g. underdeveloped testicles.

Karyogram: A drawing of a karyotype showing the band position.

Karyotype: A paired set of all the chromosomes from an individual, often banded.

Lustre: Gloss, refulgence, shining surface, iridescence.

Lymphocyte: A type of leucocyte cell formed largely in the lymph glands and spleen.

MEM-alpha medium: minimal essential medium, containing L-glutamine, ribonucleosides, deoxyribonucleosides and no sodium bicarbonate.

Metaphase: Stage in mitosis in which the chromosomes aggregate on the equator of the mitotic spindle, divide longitudinally, and the daughter chromosomes pass outward towards the poles of the spindle.

Minitab: a statistical computer package.

Mutant: see mutation.

Mutation: A change in the characteristics of an organism produced by an alteration of the hereditary material.

Orthocortical cells: Cell type present in wool fibres.

Paracortical cells: Cell type present in wool fibres.

Phenotype: The observable characteristics of an organism produced by the interaction of genes and environment.

Phytohaemagglutinin: A plant chemical derived from fungiolus beans. It acts to help stop agglutination of blood and allows blood cell growth.

Pixel: The small rectangles making up the computer screen.

Primary follicle: A wool follicle which is produced early in the developmental process. It has both a sudoriferous and a sebaceous gland present.

Recessive: A recessive characteristic is one which will not be expressed unless it is the only one present. ie in a double recessive that phenotype will be expressed, whereas a single recessive will be masked by any other character present.

Sebaceous gland: Gland which produces or contains fatty material.

Secondary follicle: A wool follicle produced in the second stage of wool development. It will contain a sebaceous gland, but no sudoriferous gland.

Sorensen's buffer: A phosphate based buffer solution.

Spindle fibre: A delicate line seen in a preparation of a dividing nucleus, and with many others making up the spindle.

S/P ratio: The ratio of secondary to primary follicles.

Sudoriferous gland: A sweat producing gland.

Trephine: An instrument of 1cm in diameter used to cut into the skin to define the region to be removed, with surgical scissors and forceps.

Tricothiodystrophy: An abnormality in humans leading to weak brittle hair with an abnormal appearance, characterised by lower than nomal cysteine content

Trypsin: Protein digesting enzyme. An endopeptidase. Found in the alimentary canal of vertebrates. Secreted by the pancreas.

T-Test: A statistical test used to determine the significance of a difference between two means.

Vips V4: A computer program used for image analysis.

Xylene: $C_6H_4(CH_3)_2$, dimethylbenzenes. There are three isomers, which cannot be separated by fractional distillation. Commercial preparation is called Xylol. Used in microscopy as a clearing agent in the preparation of specimens for embedding, and in the preparation of tissue sections for mounting.

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