

Histology of collagen in Merino sheep skin and its
association with skin wrinkle formation and
follicle curvature

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1 Introduction

Wrinkle formation in Australian Merino sheep skin is a phenomenon with serious economic and political consequences. It has long been known (Seddon, Belschner, and Mulhearn (1931) [23]) that wrinkled sheep are more susceptible to blowfly strike. The use of the *mulesing* operation to control flystrike in Merino sheep has recently been the subject of intense animal ethics scrutiny. No effective alternative management option has appeared. The most effective long term solution would seem to be to breed the wrinkle out of Merino sheep. This approach has at times met with resistance from some Australian Merino breeders who feel that the extra skin surface area of wrinkled sheep is necessary to achieve high levels of wool production. Breeding plans which include some culling on wrinkle usually do not lead to its complete elimination (for example Turner Dolling and Kennedy (1968) [27]).

This study is an attempt to go back to the basic biology of wrinkle formation, to see whether we can understand the tissue structure of a wrinkle, and to see if that suggests a better approach breeding of wrinkle-free sheep, without lowering productivity or adversely affecting wool quality.

There have been very few attempts to define what a wrinkle actually is. The early work of Carter(1943) [3] went as far as describing and naming all the folds on the neck, body, and breech, and developed a set of photographic scores for degree of wrinkle. Carter used the terms *fold* and *wrinkle* interchangeably, but he distinguished the small *pin wrinkles* present in all Merinos, from the larger folds which develop to varying degrees as the sheep matures. From this early start, there is, somewhat surprisingly, nothing on the biology of wrinkles, until the study of Mitchell et al(1984) [17].

The Mitchell et al(1984) [17] paper defines five tissue layers in sheep skin.

Layer1 epidermis is mainly keratinised protein

Layer2 contains wool follicles and accessory glands, and is part of the dermis.
Sometimes called *papillary layer*.

Layer3 layers 2 and 3 together called 'dermis'. Contains fibrous proteins, collagen, and elastin. Sometimes called *reticular layer* although the structure is not always reticular, but may be interwoven.

Layer4 contains voluntary muscle, collagen and elastin

Layer5 adipose tissue

These are illustrated in Figure 1

Only the first 3 layers curve upward in a folded section of skin, layers 4 and 5 remain straight. This can be seen in Figure 1. Mitchell et al note that Layer2 is much weaker than Layer 3 (collagen not as hard). When wrinkles or folds



Figure 1: Merino sheep skin showing layers. 1. epidermis with wool fibres; 2. papillary layer of dermis; 3. reticular layer of dermis; 4. areolar tissue and muscle; and 5. adipose tissue. Two wrinkles are present; one alongside each side of the forceps (from Mitchell et al (1984) [17])

occur in the skin, Layers 1,2, and 3 buckle up into a fold, while Layers 4-5 are straight. It appears as if wrinkles are formed either by an overgrowth of Layers 1-3, or by a shrinkage or tightening of Layer 4. Mitchell has demonstrated this by showing that if Layer 4 (and Layer 5) are dissected away from a skin specimen with wrinkles, the folds in Layers 1-3 flatten out. So in a wrinkled sheep, Layer 4 is holding the skin under some tension, which relaxes when Layer 4 is removed.

Even less is known about wrinkle development. Merino lambs are born with visible wrinkles. A somewhat obscure reference (Bogolyubsky (1940) [1]) asserts that wrinkles were observed forming in foetal skin of Karakul and Merino lambs at around 100 days of gestation. That is about the time at which the secondary derived follicles initiate. Carter(1943) [3] presents a photograph of the skin surface of a 10 day old Merino lamb (Plate 13 Figure 1) which clearly shows small *pin wrinkles*. There are no other studies of foetal wrinkle development, but there is a considerable literature on follicle development (see Fraser and Short(1960) [4] and Maddocks and Jackson(1988) [15] and Ryder and Stevenson(1968) [22] for reviews). There is some literature on collagen development in sheep skin, and we will look at that below.

What is to be investigated in this study is that the amount and type (and maybe timing and arrangement in the skin) of collagen development might be a factor involved with both wrinkle development and follicle development. So what is known about collagen? Well, it is already present in the dermis

(layers 2 and 3) of foetal skin at the time follicles develop (Knight et al (1993) [14]). These authors distinguish two collagen types (Type III or 'soft' collagen, and Type I or 'hard' collagen) and note that Type III is highest at 75 days of gestation, and falls progressively as the foetus develops, while Type I is low at day 75 and rises to over 50 percent by birth. Collagen fibres are formed from cells called *fibroblasts*. At 75-80 days the fibroblasts appear as plump, immature cells surrounded by reticular collagen fibres which are composed of Type III collagen. By birth the fibroblasts have matured and the collagen fibres may be intermeshed to varying degrees. If the fine reticular fibre pattern remains, it is soft collagen, if the fibres intermesh the collagen tissue is hardened to various degrees.

Collagen development, secondary follicle development and wrinkle formation all seem to commence at the same time of around 100 days of foetal age. Follicle development ceases at around birth (150 days) but development of collagen and wrinkles continues into the adult sheep. In this study we look at the end points of development - that is we study collagen and follicles in adult sheep with and without wrinkles. That will not reveal the details of development, but it should make clear any obvious associations between collagen, wrinkles, and follicles.

2 Materials and Methods

The experimental design was to choose, by visual inspection, individual sheep with wrinkle-free skin and wrinkly skin from each of a number of Australian Merino flocks. The flocks available for this study were mostly flocks which were undergoing breeding towards the SRS™ Merino type. Consequently most of the sheep chosen as examples of wrinkle-free sheep would have the loose and supple skin which is characteristic of SRS™ Merinos. There is another sort of wrinkle-free sheep which has low follicle density and tight skin and this type is probably not well represented in the present study.

Two trials were conducted

Trial 1 Two sheep were chosen from each of five Merino flocks, one wrinkle-free and one wrinkles. This is a randomized block design without replication . The blocks are the five flocks, and the treatment is the presence or absence of wrinkle.

Trial 2 Eighteen sheep were chosen from each of two flocks, nine wrinkle-free and nine with wrinkles. This is a randomised block design with replication. The second of these two flocks was more wrinkled and was not breeding towards the SRS™ Merino type.

2.1 Skin samples

In Trial 1 a biopsy sample was taken from the midside position on each sheep and the specimens were trimmed in the normal manner before processing, so

that only Layer 1 (epidermis) and Layer 2 (papillary dermis) were present for histological observation.

In Trial 2 , for the sheep with wrinkly skins, skin biopsies were collected from on the wrinkles as well as between the wrinkles. For the wrinkle-free sheep only one biopsy sample was collected. These specimens included Layers 1 to 4, ie only the adipose tissue was trimmed.

Midside skin samples were collected using a 10 millimetre circular trephine (Acu Punch skin biopsy punches, Acuderm, Inc.) and fixed in 10% formol saline solution.

2.2 Macroscopic skin observations

Skin samples were washed in several changes of water, the wool stubble trimmed and then examined under a magnifying lamp (x 3 magnification). Scores for suppleness (1 = hardened to 5 = supple) of the papillary layer and reticular layer were made. Each skin sample was examined to determine if layers 2 and 3, and layers 3 and 4, were free or fixed and whether localized hardening and folding of the skin had occurred.

The thicknesses of the papillary dermis and the reticular dermis were measured using a ruler graduated in one millimetre divisions. A Mitutoyo ballpoint gauge (model no. 2046S) was then used to measure the compressed thickness at four sites for each skin sample.

2.3 Histological skin processing and observations

2.3.1 Collagen observations

Skin samples used for haematoxylin and eosin staining (H-E) and picrosirius red (PSR),were fixed in 10% neutral buffered formalin for 24 hours before being processed to wax in an automated tissue processing platform (Shandon Excelsior, Thermo Scientific, USA), and then embedded in paraffin wax. Four micron sections were cut and placed onto slides for H-E staining for tissue morphology. Serial section was also employed on a separate slide for PSR staining to highlight collagen content. Staining was performed manually.

Sections were then reviewed microscopically (BX53 Olympus, Australia)), and images taken on 3 CCD camera (DP72, Olympus, Australia) under both bright field and polarized conditions for PSR staining.

For PSR collagen analysis, the 40x objective was employed at a fixed exposure to take high power images of 5 random deep dermal fields of view for computational analysis.

[Sanaz it seems to me that these 5 random fields would have been chosen within the red stained areas with collagen present.
I think we should say so]

The images for each sample were then uploaded for quantitative analysis via the ImagePro Plus (Media Cybernetics, USA) 7.1 software in which thresholds

were set to count all pixels comprising of the red staining fibres in the PSR stained specimen against the total pixels. A mean was calculated for each of the specimens' 5 images and graphed.

Polarised light was employed in order to try and determine the type of collagen present within each of the samples.

[Sanaz, you made a comment about this on Jim's last draft.

The yellow and green reflectances are likely to indicate soft (Type III) collagen (Sanaz, please check this statement).

Need to be careful here Jim, as no one has been able to definitively prove the birefringences of PSR staining with collagen fibres, and some of the literature contradicts itself.

I can pull a few papers to reference as a guide to the reviewers?

and this

Birefringence measurements of PSR stained skin sections indicate that nearly all (. %) of the collagen sheets in the subfollicular layer of the papillary dermis have the deep red light reflectance indicative of hard (type I) collagen. (Sanaz, please check this statement).

Again Jim, we have to tread carefully here making definitive statements based on colour birefringence. We can certainly point out that the thicker fibres were red, and the thinner fibres more green, with some yellowish-orange colours in between.

Can you make some statement that is either definitive or indicative and give a reference please?

I think it belongs here in the Methods, not mixed up with Results where Jim had it.

2.3.2 Vertical skin sections

Vertical skin sections, approximately 0.3 millimetres wide, were cut freehand with a sharp razor blade on a freezing stage and stained with 0.25 % Nile blue sulphate, as described by Nay (1973). The sections were cut parallel with the angle of emergence of the fibres to avoid cutting through follicles. Mean follicle curvature was scored from 1 = straight follicles to 7 = tangled follicles by reference to a set of standard drawings used by Nay and Johnson (1973). Follicle depth was measured as both the perpendicular and angular distances (in millimetres) between the skin surface and the lower ends of the follicle bulbs, along with follicle bending, as described by Maddocks and Jackson (1988).

2.3.3 Horizontal skin sections

Horizontal skin sections were also prepared as described by Maddocks and Jackson (1988) using the frozen section technique and measurement procedures of

Nay (1973). The sections were used to measure follicle density, secondary follicle to primary follicle ratio (S/P ratio), primary fibre diameter and secondary fibre diameter of the sheep.

JW to describe measurement of orientation of follicle groups and measurements made of collagen sheets in subfollicular layer of papillary dermis.

2.4 Summary of measurements

2.5 Statistical Methods

Data were imported into the R statistical program [21] and analysed using the *aov()* function for analysis of variance.

3 Results

We follow the path of looking first at overall morphology of skin specimens, then at the details of collagen structure, and finally at other related measurements

3.1 Macro observations on biopsy specimen

In Table 1 we present the suppleness scores and percent compressibility of specimens from the sheep from Trial 1.

Table 1: Suppleness scores and compressibility measurements for Flocks 1 to 5 of Trial 1

Flock No.	Sheep No.	Skin Type	Suppleness Score	Compressibility Percent
1	W206	Wrinkle-free	5	75
1	W205	Wrinkled	2	54
2	W490	Wrinkle-free	5	64
2	W479	Wrinkled	2	39
3	W555	Wrinkle-free	5	67
3	W547	Wrinkled	1	58
4	W567	Wrinkle-free	5	70
4	W558	Wrinkled	2	63
5	W283	Wrinkle-free	5	69
5	W290	Wrinkled	2	44

We see that the wrinkled sheep specimens were consistently less supple and less compressible than those of the wrinkle-free sheep. These differences in Suppleness and Compressibility were tested for significance in an analysis of variance shown in Table 2

Table 2: Analysis of variance of Suppleness score and Compressibility

Response Suppleness		Df	Sum Sq	Mean Sq	F value	Pr(>F)
FlockNo		4	0.40	0.10	1.00	0.5000
SkinType		1	25.60	25.60	256.00	0.0001
Residuals		4	0.40	0.10		
Response Compressibility		Df	Sum Sq	Mean Sq	F value	Pr(>F)
FlockNo		4	305.60	76.40	1.99	0.2608
SkinType		1	756.90	756.90	19.71	0.0113
Residuals		4	153.60	38.40		

The differences between skin types (wrinkled and wrinkle-free) were significant for both Suppleness and Compressibility. Flock differences were not significant.

3.2 Skin tissue Morphology

The pairs of wrinkle free and wrinkled sheep from each flock in Trial 1 showed consistent visual differences in their tissue structure. Figure 2 shows vertical sections stained with H-E from the wrinkled and wrinkle-free pair of sheep from flock 2.

The wrinkled sheep has a greater amount of staining in the connective tissue in the lower dermis below the deepest follicle bulbs and to some extent in between the deepest bulbs. The staining also seems to be in larger clumps in the wrinkled sheep, whereas in the wrinkle-free sheep the lower dermal connective tissue has a finer more regular structure.

The follicles in the wrinkled sheep are at a variety of angles and are curved, as evidenced by the follicle shafts being sectioned and the follicle bulb being elliptical indicating sectioning at an angle. In contrast in the wrinkle-free sheep the sectioned follicles are more uniform.

These differences were consistent across all sheep.

Although the Trial 2 biopsy samples were not trimmed before sectioning, the specimens displayed in Figure 2 do not show any layers below Layer 3. We need to see what connective tissue is present in Layer 4 (muscle layer).

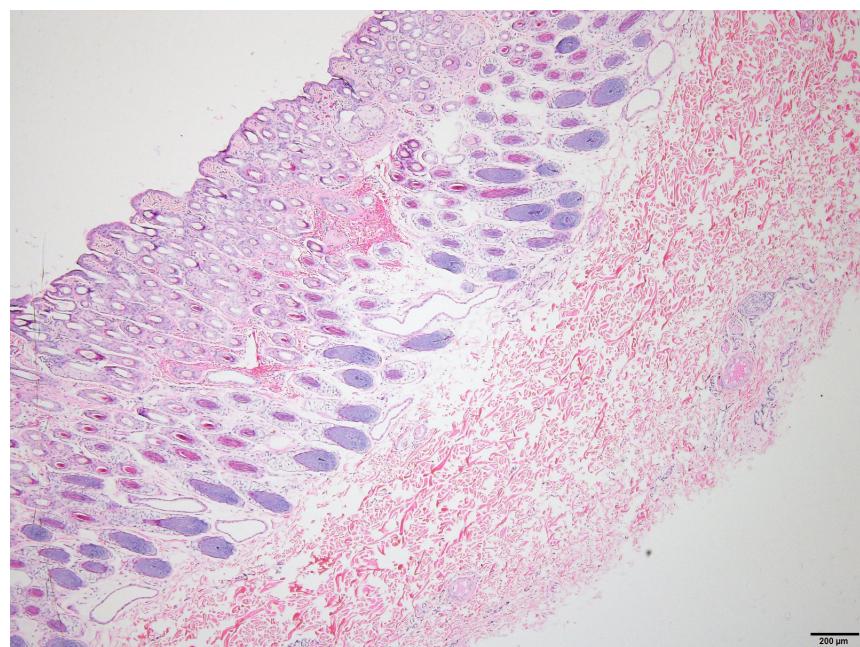
Sanaz, do you know why these sections have Layer 4 trimmed ?
They are from Trial 2, and should be untrimmed.

The only images of untrimmed samples that I could find were the two below which came to me from Jim by email. There are none in his computer files which Sally retrieved for me?

We can check on this by looking at a specimen , which was not trimmed



(a) Plate (i) Sheep 3437 Wrinkled



(b) Plate (ii) Sheep 3457 Wrinkle-free

Figure 2: Vertical sections from a wrinkled (i) and a wrinkle-free (ii) sheep from Trial 2 flock 1 stained with H-E.

before sectioning. Figure 3 shows one example section which is from a wrinkled sheep from Flock 1 of Trial 2.

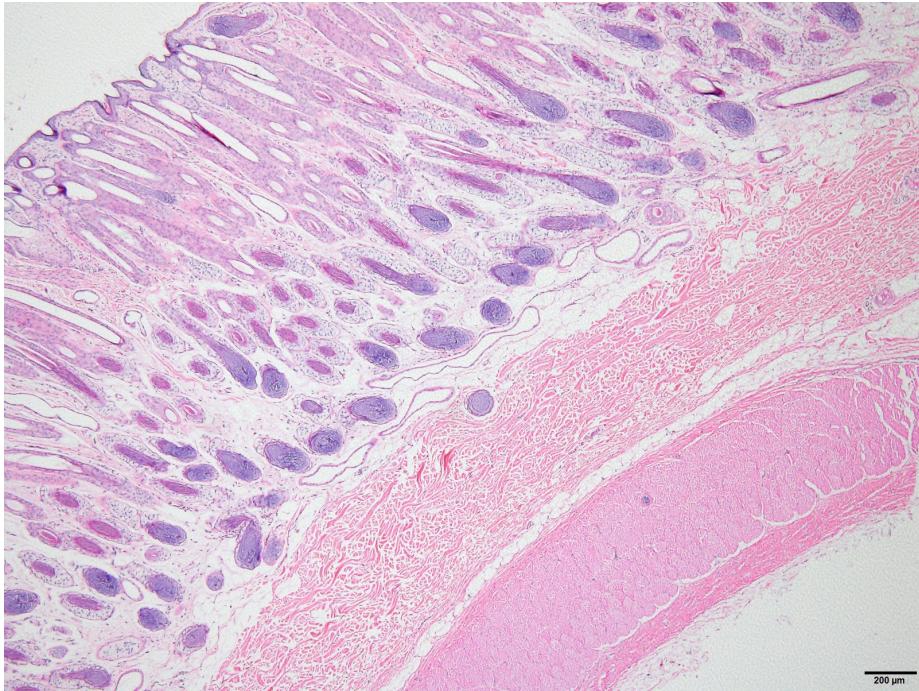


Figure 3: Vertical section from a wrinkled sheep (3456) from Trial 2 Flock 1 stained with H-E. This section is from an untrimmed biopsy specimen and shows all 4 layers (Epidermis, Papillary dermis, Reticular dermis and Muscle layer).

It is clear from Figure 3 that the connective tissue in Layer 3 (lower or reticular dermis) does not extend further into Layer 4 (muscle layer). The muscle layer has only stained with the pink eosin counterstain and does not show the reticular structure of connective tissue, except perhaps for thin bands above and below the muscle mass.

We are therefore concerned with the nature of this connective tissue in the lower dermis only. We wish to quantify and qualify the way in which it differs between wrinkled and wrinkle-free sheep.

3.3 Detailed morphology of connective tissue

The stain picosirius red (PSR) was used to differentiate collagen from other components of connective tissue. Figure 4 shows a section from the same sheep as Figure 3 examined with normal bright field microscopy.

The collagen is red. There is some collagen showing in the Layer 3 (papillary dermis), a dense band of collagen in Layer 3 (subpapillary dermis), and Layer 4 consists of yellow stained muscle tissue with a band of red collagen above



Figure 4: Vertical section from a wrinkled sheep (3456) from Trial 2 Flock 1 stained with PSR and viewed with bright field microscope. This section is from an untrimmed biopsy specimen and shows all 4 layers (Epidermis, Papillary dermis, Reticular dermis and Muscle layer). Collagen (stained red) is present in Layers 2 and 3, and on the borders of Layer 4

and below it. Fibres and follicle bulbs are stained yellow by the picric acid component of PSR.

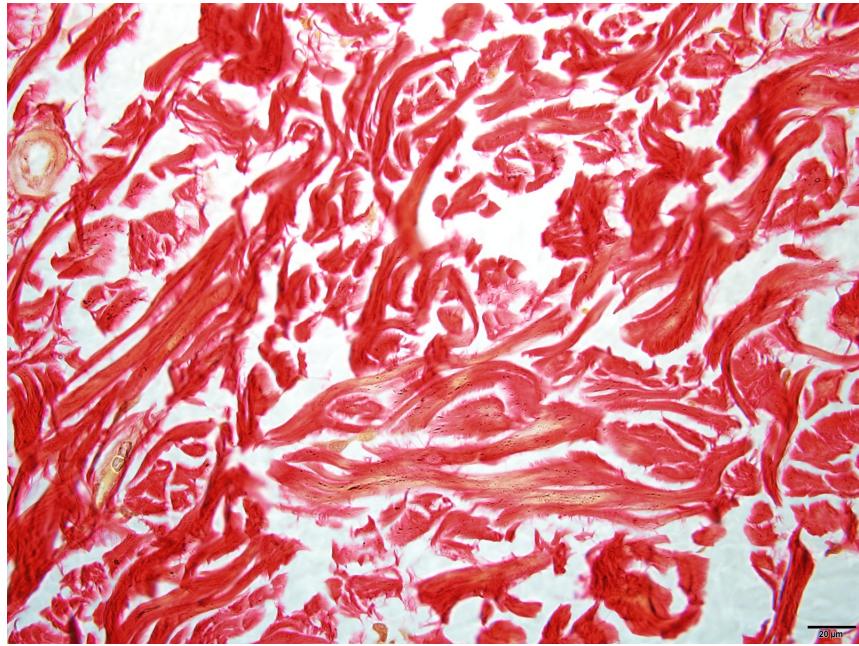
So we can conclude that the connective tissue in Layer 3 of wrinkled sheep contains collagen.

3.3.1 Amount of collagen

Since the nature of the connective tissue in Layer 3 is what seems to differ between wrinkled and wrinkle-free sheep, we attempt to quantify the difference in amount of collagen to see if this explains the observed difference in appearance.

To quantify the amount of collagen in Layer 3, 5 fields under a 40x objective were chosen at random from within Layer 3 (reticular dermis) of each PSR stained section from each sheep in Trials 1 and 2. A typical image from one field of a wrinkled and a wrinkle-free sheep is shown in Figure 5.

The two fields shown in Figure 5 were chosen to be typical of the difference between wrinkled and wrinkle-free sheep. They show that the collagen in Layer 3 of wrinkled sheep is in larger aggregates and suggest that there is more collagen



(a) Plate (i) Sheep 3437 Wrinkled



(b) Plate (ii) Sheep 3457 Wrinkle-free

Figure 5: Fields chosen at random from within Layer 3 (subpapillary dermis) stained with PSR and viewed with a 40x objective.

present (as evidenced by more red stained areas) in wrinkled sheep. We set out to confirm this with some quantitative data.

The image analysis procedure described in the methods was used to assess the total amount of red stained pixels in each field. What was actually calculated was the sum of the calibrated optical densities of all the pixels in the red image. The integrated (ie summed over all pixels) red pixel optical density for each sheep is shown in Figure ?? for Trial 1 and Figure ?? for Trial 2. The total number of pixels in an image taken with a 40x objective was 1920000, so one could scale these optical density sums to the average optical density of a pixel by dividing by 1920000.

The significance of the differences apparent in Figure ?? was tested by analysis of variance extracting terms for FlockNo, SkinType, and their interaction, as shown in Table 3.

Table 3: Analysis of variance of red pixel optical density sums for Trial 1

Source	Df	Mean Square	F value	Significance
FlockNo	5	34266275526.60	1.688	NS
SkinType	1	259320673232.61	12.775	*
FlockNo:SkinType	5	20298859594.97	12.934	***
Residuals	48	1569402773.43		

Signif. codes: *** 0.001 ** 0.01 * 0.05

The residual term in Table 3 is the variation between randomly chosen Fields within a specimen, because there are no replicate sheep within each Flock:SkinType subclass. The difference between the wrinkled and wrinkle-free SkinTypes is shown to be significant but only at the 5% level. The Flock differences are not significant, and there is an interaction.

The equivalent analysis of variance for Trial 2 (Figure ??) data is shown in Table 4.

Table 4: Analysis of variance of red pixel optical density sums for Trial 2

Source	Df	Mean Square	F value	Significance
FlockNo	1	91548864255.47	4.14	NS
SkinType	1	412338258758.73	18.67	***
SampPos	1	50325174039.48	2.28	NS
FlockNo:SkinType	1	101676878442.96	4.60	*
FlockNo:SkinType:SheepNo	49	22076924782.32	9.02	***
Residuals	218	2447351456.99		

Signif. codes: *** 0.001 ** 0.01 * 0.05

The difference between wrinkled and wrinkle-free SkinTypes is now shown to be highly significant. The Flock differences were not significant and there is a significant interaction of Flock with SkinType.

The on-wrinkle and between-wrinkle sampling positions within the wrinkled specimens were not significantly different . The on-wrinkle specimens actually had a lower integrated optical density than the between-wrinkle specimens indicating slightly *less* collagen on a wrinkle than between wrinkles.

There was also a significant amount of variation between sheep within the FlockNo and SkinType combinations. Sheep are much more variable than image Fields within a sheep, which is what the Residual term in Table 4 represents. In this analysis the Sheep term is the error term for all terms above it in the analysis of variance table, whereas in Trial 1 there was no sheep replication and we were forced to use the FlockNo:SkinType term as the error. This explains why the SkinType differences were less significant in Trial 1.

The actual means and their standard errors for integrateds optical density for both Trial 1 and Trial 2 are shown in Table 5

Table 5: Means and standard errors for integrated red pixel optical density of wrinkled and wrinkle-free sheep in Trial 1 and Trial 2

Trial	Parameter	Wrinkle-free	Wrinkled (between-wrinkle)	Wrinkled (on-wrinkle)
1	Mean	83748	215232	
	Standard deviation	47535	98720	
2	Mean	280851	380427	347170
	Standard deviation	70609	75988	96787

We see that the wrinkle-free sheep actually have a very low amount of collagen in Trial 1. This is probably because the Trial 1 sheep were selected from SRS-Merino stud flocks and those chosen as wrinkle-free were likely to be extreme examples of wrinkle-free sheep, of a type not found in normal Merino flocks.

The Trial 2 sheep are the opposite. These were from commercial flocks, and here the wrinkled sheep (either the on-wrinkle or between wrinkle specimens) had a higher amount of collagen than the wrinkled sheep from Trial 1. Again, this simply reflects the fact that more extreme wrinkled sheep were available for selection in rial 2.

The standard deviations were greater for wrinkled sheep, and greater for on-wrinkle than between-wrinkle specimens. Apparently wrinkled skins are more variable, at least in their collagen density. We did not test whether these differences in standard deviation were significant.

There are some questions hanging over these data.

The data can not be pixel counts, because they are not integers.
 They must be something like counts weighted by the intensity of redness
 of each pixel, or something similar.
 Sanaz, can you please find out exactly what the data are?

3.3.2 Size of collagen fibrils

3.3.3 Type of collagen

3.4 Follicle characteristics

A number of follicle attributes seem to differ between wrinkled and wrinkle-free sheep. These are documented below

3.4.1 Follicle curvature scores

Follicle curvature scores were available for Trial 1. The scores for each sheep are shown on Table 6

Table 6: Follicle curvature scores Flocks 1 to 5 of Trial 1

Flock No.	Sheep No.	Skin Type	Follicle Curvature Score
1	W206	Wrinkle-free	3
1	W205	Wrinkled	6
2	W490	Wrinkle-free	4
2	W479	Wrinkled	6
3	W555	Wrinkle-free	3
3	W547	Wrinkled	7
4	W567	Wrinkle-free	3
4	W558	Wrinkled	3
5	W283	Wrinkle-free	2
5	W290	Wrinkled	7

In each case except for Flock 4, the wrinkle-free sheep had a lower follicle curvature score than the wrinkled sheep.

An analysis of variance of these scores is given in Table 7

Table 7: Analysis of variance of follicle curvature score for Trial 1 data

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
FlockNo	1	0.80	0.80	0.47	0.5165
SkinType	1	19.60	19.60	11.43	0.0117
Residuals	7	12.00	1.71		

This shows that the difference between wrinkle-free and wrinkled sheep was significant at the 1 percent level.

3.4.2 Follicle curvature measurements

Follicle curvature measurements were made for Trial 2. There is a document detailing measurement methods and their statistical analysis available in Watts and Jackson (2018) [29].

Here we present only a summary of the results. Table 8 shows means for follicle depth, straight length of the follicle, curved length of the follicle and radius of curvature.

Table 8: Means for follicle measurements separately for each Flock and each Skintype for Trial 2

Flock	Skin.type	Folldepth	Straightlen	Curvlen	Radcurv
1	wrinkle-free	1.579	1.827	1.839	6.97
2	wrinkle-free	1.721	1.846	1.854	8.99
1	wrinkled	1.972	2.136	2.222	2.92
2	wrinkled	1.833	1.925	2.035	2.15

All four measurements differed between wrinkled and wrinkle-free sheep, and the differences were significant for all four traits.

3.4.3 Follicle density and S/P ratio

3.4.4 Follicular degeneration

Dr Watts saw evidence of follicle degeneration in wrinkled sheep. His statement was

” I am happy that there is not only follicular distortion caused by collagen but also follicular degeneration. I can see evidence of bent follicle bulbs right at the tip of where the follicles are curving more or less at right angles. I can also see in these affected sheep, the wrinkly skinned ones, that the follicle bulb cells are becoming vacuolated ie. undergoing cellular degeneration. The fibre defects we are encountering appear to be the consequence of this follicle degeneration.”

There are no measurements to support this statement. The fibre defects referred to are fibre naps.

4 Discussion

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