

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) [29]) 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) [34]).

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) [22].

The Mitchell et al(1984) [22] 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) [22])

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) [5] and Maddocks and Jackson(1988) [20] and Ryder and Stevenson(1968) [27] 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) [16]). 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 six 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 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 image analysis aimed at determining the amount of collagen in each field.

The five 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 field. This provided a measure of the area of the field occupied by red stained collagen fibres.

A slightly better measure of the total amount of collagen in the field could be obtained by allowing for the intensity of red staining of each pixel. This would be a measure density of collagen within the pixel and the depth of collagen through the thickness of the section. The greyvalues for each pixel were converted to optical density, and the optical densities summed (ie integrated) over all pixels in the field. A mean was calculated for each specimen, averaged over 5 fields, and graphed. The optical density data for each field subjected to analysis of variance to test for differences between wrinkle-free and wrinkled sheep, and, in Trial 2, to test for differences between on-wrinkle and between-wrinkle specimens within the wrinkled sheep. All specimens were measured in this way, and this is one of the main quantitative results of the study.

Polarised light was employed in order to try and determine the type of collagen present within each of the samples. Collagen fibres stained with PSR have enhanced birefringency compared to that in H-E stained sections (Junqueira et al(1979) [14]). Under polarised light they show coloured red, orange, yellow, or green, in order of thickness of the bundles of fibres. Thus red or orange would indicate Type I or hard collagen (which has thick bundles of fibres) while yellow or green would indicate Type III collagen which has individual fibres in a net-like structure.

Attempts to use polarised light images to make quantitative assessments of the amounts of each Type of collagen have been criticised in the literature (Lattouf et al (2014) [18]). The main issue seems to be that the birefringence is directional, only the fibres aligned with the direction of polarisation will show colours. We refrained from attempting this quantification, so our polarised light results are only qualitative.

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 [26] and analysed using the *aov()* function for analysis of variance. Allowance was made for the subsampling design by choosing an appropriate error level for the F tests in 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	S558	Wrinkled	2	63
5	Z529	Wrinkle-free		
5	Z530	Wrinkled		
5	S283	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).

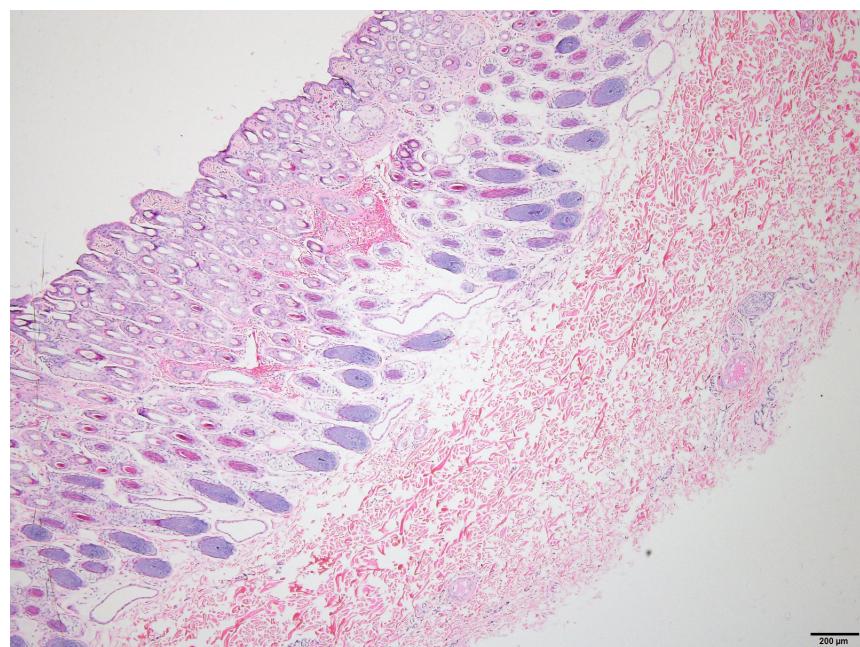
Sanaz, do you know why these sections have Layer 4 trimmed ?
They are from Trial 2, and should be untrimmed.

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.

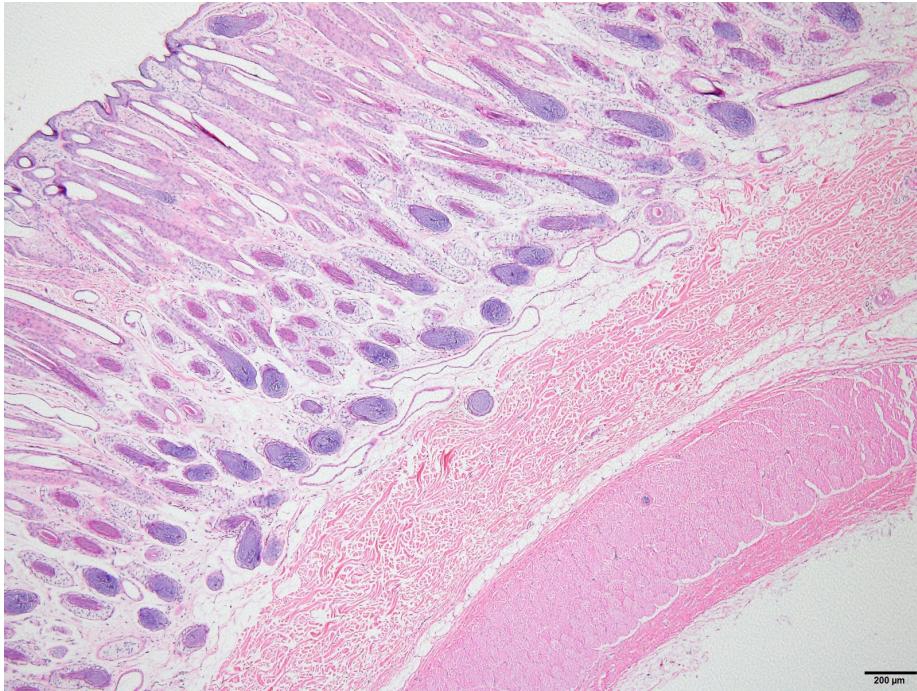


Figure 3: Vertical section from a wrinkled sheep (3456) from Trial 2 Flock 1 stained with H-E. This section is from an untrimmed biopsy specimen and shows all 5 layers (Epidermis, Papillary dermis, Reticular dermis, Areolar tissue and Muscle layer, and Adipose tissue).

It is clear from Figure 3 that there is connective tissue in Layer 3 (lower or reticular dermis), then a layer which may be muscle and/or connective tissue, then a wider layer of adipose tissue, and finally another layer of muscle and/or connective tissue. The muscle layer(s) have only stained with the pink eosin counterstain and do not show the reticular structure of connective tissue.

We are concerned with the nature of the 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 picrosirius 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 stained red. There is some collagen showing in the Layer 2 (papillary dermis), a dense band of collagen in Layer 3 (subpapillary dermis),



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 5 layers (Epidermis, Papillary dermis, Reticular dermis and the Fat/Muscle layers). Collagen (stained red) is present in Layers 2 and 3, and on the borders of the Adipose layer

and Layers 4 and 5 consist of yellow stained adipose tissue with a band of dense red collagen above and below it. These two bands are muscle tissue which contains both collagen and elastin fibrils. Wool fibres and follicle bulbs are stained yellow by the picric acid component of PSR. Within the adipose tissue layer there are tiny tracks of red stained connective tissue, presumably between the fat cells.

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 (bundles of collagen fibres), and suggest that there is more collagen 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 6 for Trial 1 and Figure 7 for Trial 2.

These data are intended as a measure of the total amount of collagen tissue present in the microscope section at the position of the chosen field in the lower dermis. 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. We chose not to do this scaling. We can see that for Trial 1 the wrinkle-free sheep always had less collagen, except for those in Flock 4. In Trial 2 the wrinkle-free sheep always had less collagen than the between-wrinkle sample from the wrinkled sheep, but the on-wrinkle sample was more variable.

The significance of the differences apparent in Figure 6 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

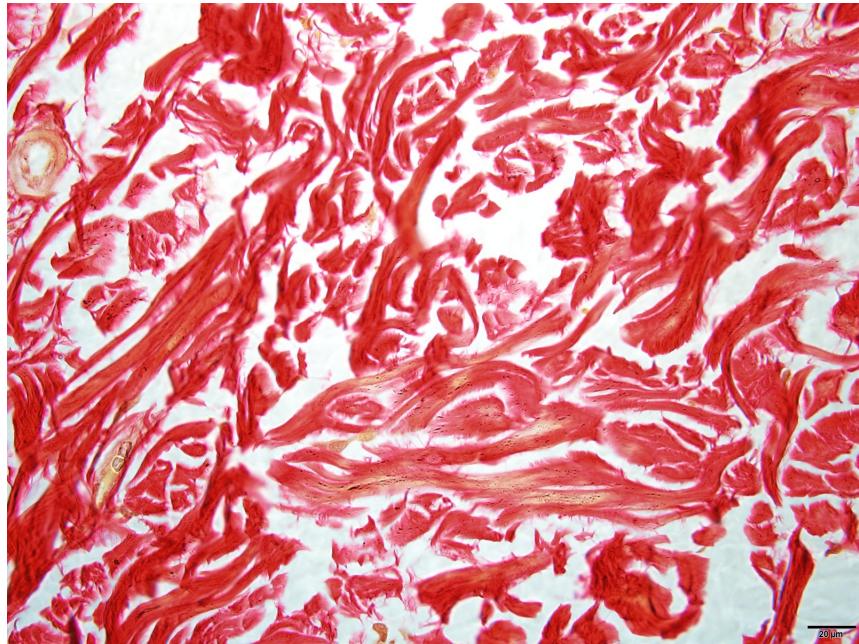
Source	Df	Mean Square	F value	Significance
FlockNo	5	34266275526.60	1.688	NS
SkinType	1	259320673232.61	12.775	*
FlockNo:SkinType	5	20298859594.97	12.934	***
Residuals	48	1569402773.43		

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 7) data is shown in Table 4.

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.



(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.

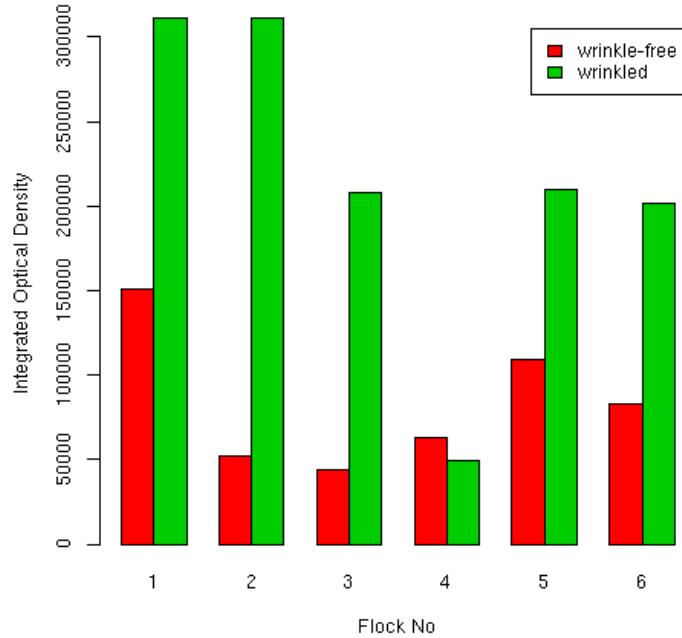


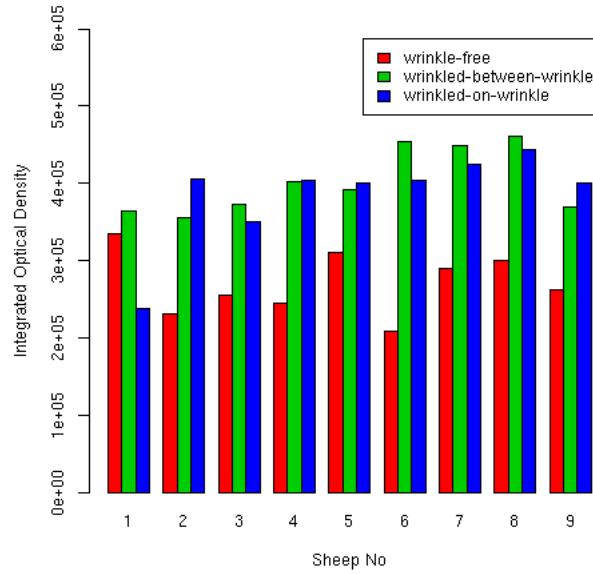
Figure 6: Integrated optical density of the red images of sections stained with PSR for each sheep in Trial 1 averaged over five microscope fields

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.

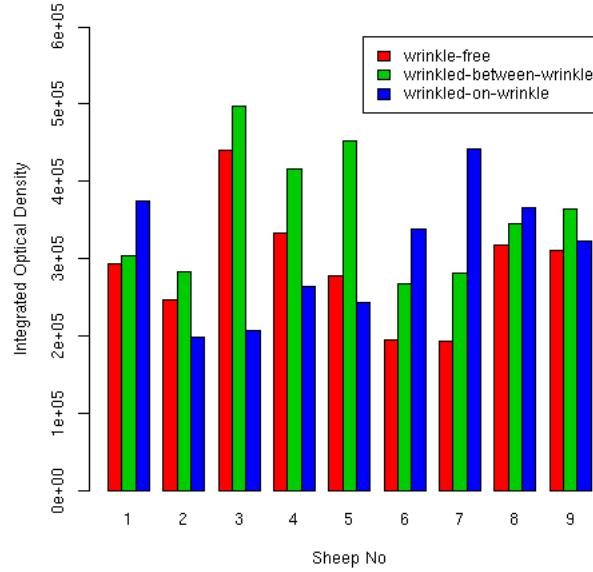
There was also a significant amount of variation between sheep within the FlockNo and SkinType combinations. Sheep are much more variable than image Fields within a sheep, which is what the Residual term in Table 4 represents. In this analysis the Sheep term is the error term for all terms above it in 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 deviations for integrated optical density for both Trial 1 and Trial 2 are shown in Table 5

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.



(a) Subfigure (i) Flock No 1 of Trial 2



(b) Subfigure (ii) Flock No 2 of Trial 2

Figure 7: Integrated optical density of the red images of sections stained with PSR for each of the nine sheep in each Flock of Trial 2, averaged over five microscope fields

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

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	
	N	6	6	
2	Mean	280851	380427	347170
	Standard deviation	70609	75988	96787
	N	18	18	18

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.

The data and analyses show that there is more collagen in the lower dermis of wrinkled sheep than wrinkle-free sheep. The actual size of the difference varied from 2.5 x in Trial 1 to 1.4 x in Trial 2. Within wrinkled sheep there was no difference in amount of collagen between samples taken on a wrinkle or between wrinkles.

3.3.2 Spatial location and structure of collagen

We have shown that there is more collagen in wrinkled sheep. We need to look and see if it occupies the same part of the dermis. We also need to investigate whether the arrangement of collagen fibres varies.

The best images for these purposes were taken with a 10x objective. Figure 8 shows images of layers 2 and 3 in specimens from two sheep, one being wrinkled (a between-winkle specimen) and one being wrinkle-free.

The most obvious difference is that in the wrinkled sheep specimen the collagen extends up into the follicular region, there being conspicuous amounts of collagen in and around the follicle bulbs. In the wrinkle-free sheep there is very little amount of collagen in amongst the follicle bulbs, and the collagen immediately below the bulbs is fine structured and presumably of a reticular type.

There is also a difference in structure. In the wrinkled sheep there are large pieces of very dense collagen (judging by the intensity of staining) in the lower dermis, and amongst the follicles. These large entities are presumably bundles of collagen fibrils. In the wrinkle-free sheep there are some dense pieces of collagen (more further down in the dermis) but these are not as dense and not as large and not as numerous as in the wrinkled specimen. If we look back to the PSR stained images of Figure 5, these observations are confirmed. We must note that we are viewing thin (4 micron) sections. For bundles of collagen to show as large continuous areas in these sections they must align with the direction of sectioning. Hence we see some fibre bundles that have been sectioned across, and these show as small entities, and some that have been sectioned along and these show as large entities. There are fewer large entities in the wrinkle-free specimens in both Figures 5 and 8. This difference is even discernable in the images viewed with a 4x objective shown in Figure 2

We were not able to quantify these observations. The examples we have shown are from Trial 2, but the differences were consistent across both trials.

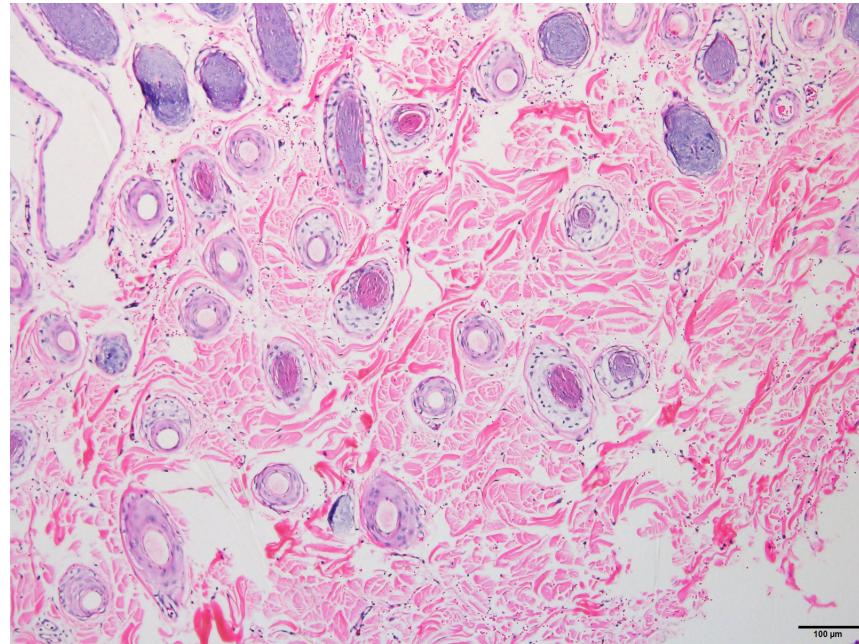
3.3.3 Type of collagen

The types of collagen relevant to skin are

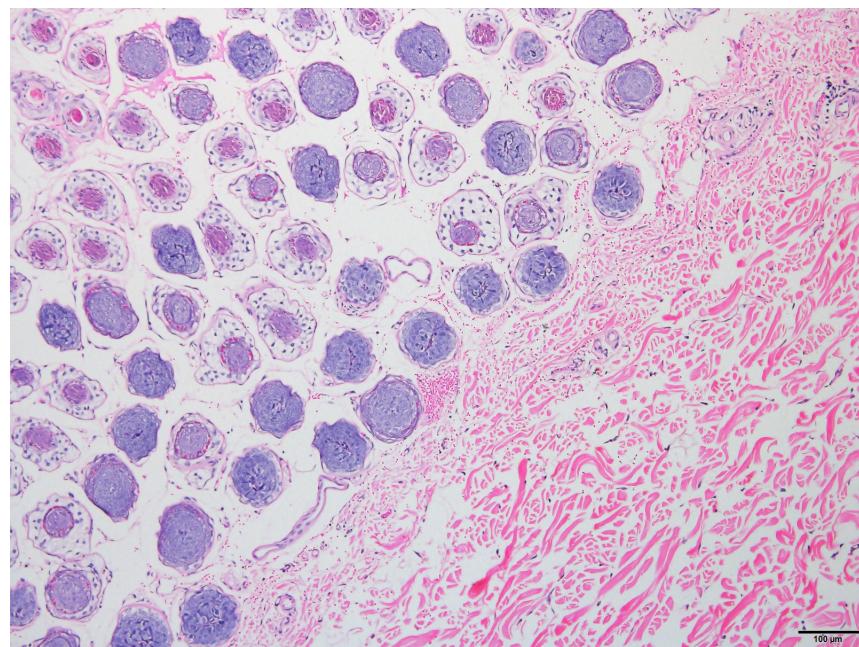
Type I or *hard* collagen forms thick bundles of eosin staining fibres. Is present in tendons, ligaments, and scar tissue. It binds things together.

Type III or *soft or reticulate* collagen forms thin separate eosin staining fibres which crosslink to form a fine mesh network supporting soft tissues. It often occurs with Type I.

Both types occur together in skin. The strength, elasticity and flexibility of skin come from the presence of collagen and elastin fibres, and presumably variations in these properties would derive from variations in the amounts and proportions of these types. For example changes in skin with ageing are partly a result of reduction in Type I collagen.



(a) Plate (i) Sheep 3453 Wrinkled



(b) Plate (ii) Sheep 3458 Wrinkle-free

Figure 8: Vertical sections from a wrinkled (i) and a wrinkle-free (ii) sheep from Trial 2 flock 1 stained with H-E. and viewed with a 10x objective.

One can distinguish the two Types of collagen simply from the size of the fibrils. For example in the PSR stained images of Figure 5 the wrinkled specimen clearly has large bundles of fibrils and therefore a considerable amount of Type I collagen. The wrinkle-free specimen, however has fewer bundled fibrils, and lots of thin individual fibrils. This is also quite obvious in the H-E stained sections of Figures 8 and 2.

There is a somewhat contentious technique referred to in the Methods section, which uses polarized light microscopy to attempt to differentiate Type I from Type III collagen. The collagen fibres are birefringent and it is asserted that they show coloured red, orange, yellow, or green on order of thickness of the bundles of fibrils. Thus, red and orange would be likely to indicate Type I collagen, and yellow and green would be likely to indicate Type III collagen.

Figure 9 shows two polarized light images under a 4x objective comparing a wrinkled sheep with a wrinkle-free sheep.

The same sections viewed under normal bright field microscopy are shown in Figure 10.

It can be seen that while both the wrinkled and wrinkle-free specimens have some lower dermal collagen (stained red with PSR stain in Figure 10), only the wrinkled specimen shows orange/red birefringence under polarised light (Figure 9).

So, there is the proof that wrinkled sheep do not just have more collagen, but the extra collagen is Type I (hard). The wrinkle-free sheep apparently has only Type III (reticular) collagen. This confirms what we concluded in the previous section from looking at the size of collagen fibre bundles.

3.3.4 Fibroblasts

The dermal cells which produce collagen fibrils are called *fibroblasts* or mesenchymal stem cells. Fibroblasts also produce reticular fibres, and the pre-papilla cells that aggregate during follicle formation.

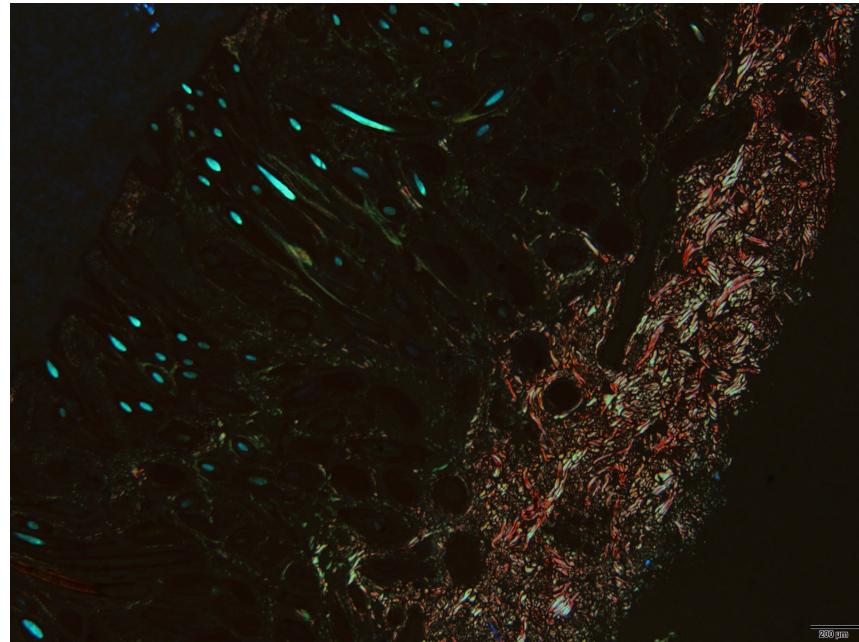
Some differences were observed in the fibroblasts in wrinkled and wrinkle-free sheep. We are only able to report Dr Watt's observation

"The dermal fibroblasts in the wrinkled sheep appear to be mature, slender fibroblasts (fibrocytes) with dark staining nuclei whereas wrinkle-free sheep have immature, plumb fibroblasts with pale staining nuclei. The density of fibroblasts in the wrinkled sheep also appears to be higher than in wrinkle-free sheep"

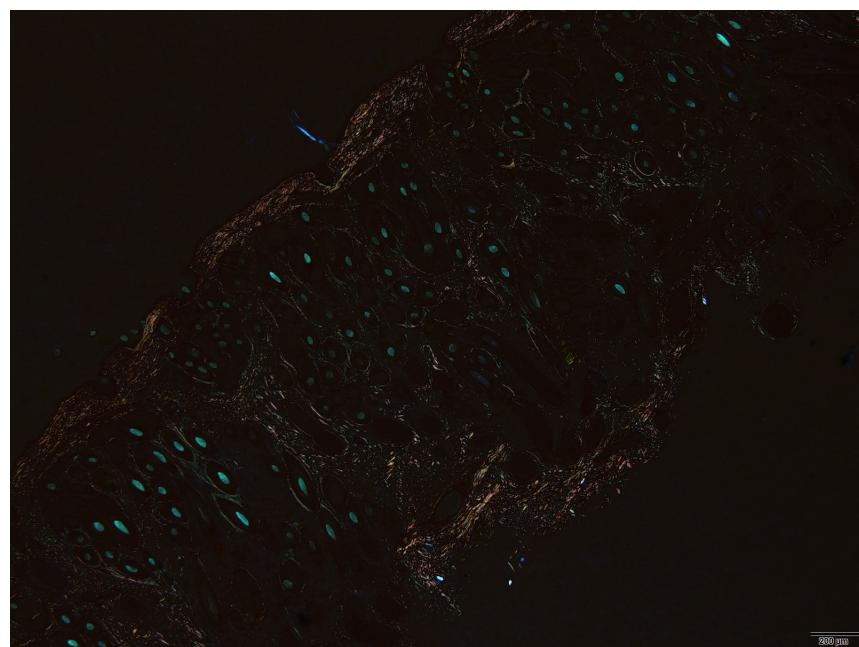
Further observations would be required to confirm this. If correct it would mean that the dermal collagen of wrinkled sheep was under more active development at the time of sampling.

3.4 Follicle characteristics

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

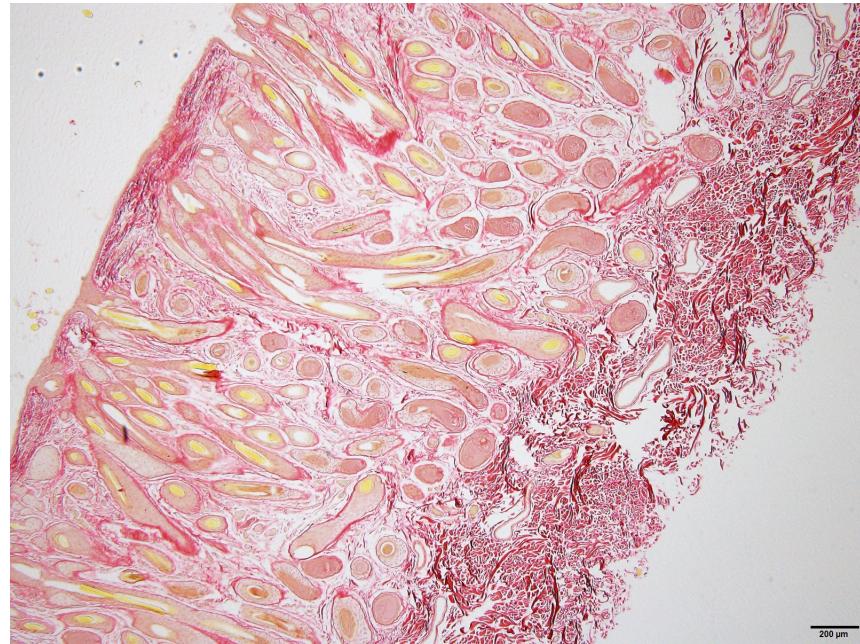


(a) Plate (i) Sheep w479 Wrinkled

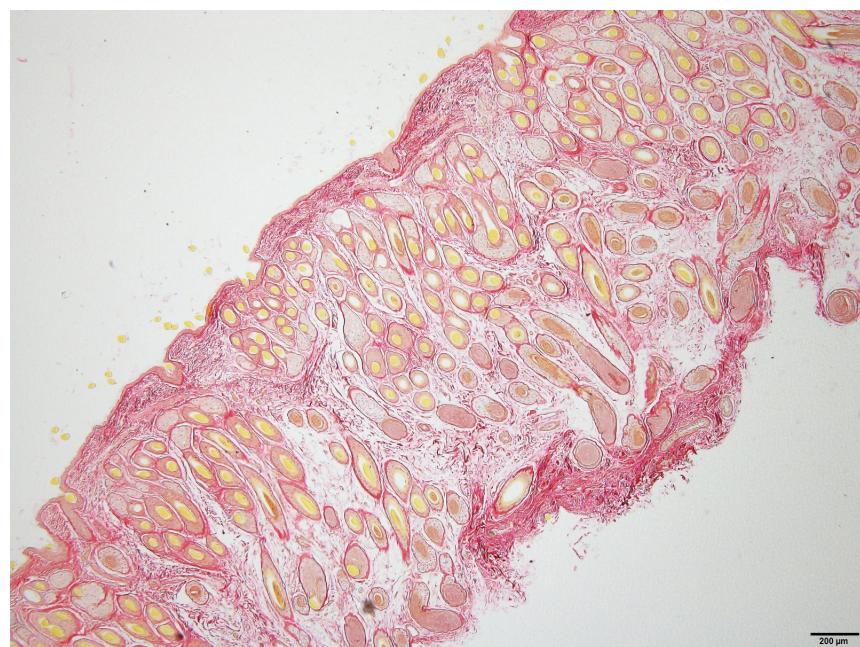


(b) Plate (ii) Sheep w490 Wrinkle-free

Figure 9: Vertical sections from a wrinkled (i) and a wrinkle-free (ii) sheep from Trial 1 flock 2 stained with PSR and examined with polarised light and a 4x objective.



(a) Plate (i) Sheep w479 Wrinkled



(b) Plate (ii) Sheep w490 Wrinkle-free

Figure 10: Vertical sections from a wrinkled (i) and a wrinkle-free (ii) sheep from Trial 1 flock 2 stained with PSR and examined with a 4x objective. These are the same two sections as shown with polarised light in Figure 9.

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) [36].

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.

All four measurements differed between wrinkled and wrinkle-free sheep, and the differences were significant for all four traits. There is a comprehensive analysis of these follicle curvature measurements in Watts and Jackson(2018) [36]. The important result is that the wrinkled sheep had a much smaller radius of

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

curvature, meaning that the follicles were more curved in wrinkled sheep. This confirms the subjective follicle curvature scores, which were for Flock 1 of Trial 2 3.8 for wrinkled sheep and 1.7 for wrinkle-free sheep, and for Flock 2 of Trial 2 5.1 for wrinkled sheep and 2.7 for wrinkle-free sheep (means of 9 sheep in each case).

The follicles of wrinkled sheep were also slightly deeper and longer.

3.4.3 Follicle density and S/P ratio

For Trial 2, some further measurements of the follicle and fibre characteristics were available, and are shown in Table 9

Table 9: Means for follicle density and fibre diameter measurements separately for each Flock and each Skintype for Trial 2

Flock	Skin.type	Follicle density	S/P ratio	Dp	Ds
1	wrinkle-free	83.9	27.8	16.4	18.4
2	wrinkle-free	94.9	27.8	17.7	18.4
1	wrinkled	76.7	21.8	18.8	20.7
2	wrinkled	66.8	22.6	20.5	20.7

Wrinkled sheep had lower follicle density, lower S/P ratio, and coarser primary and secondary fibres.

Analyses of variance (Table 10) confirmed that these differences between wrinkled and wrinkle-free sheep were significant. The Flock differences were not significant.

3.4.4 Follicular degeneration

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

Table 10: Analyses of variance of Follicle characteristics and Fibre diameters

Response Follicle no.					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Flock	1	16.75	16.75	0.06	0.8122
SkinType	1	2628.96	2628.96	9.00	0.0052
Residuals	32	9347.23	292.10		
Response S/P ratio					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Flock	1	2.86	2.86	0.24	0.6295
SkinType	1	275.42	275.42	22.83	0.0000
Residuals	32	386.11	12.07		
Response Dp					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Flock	1	19.15	19.15	3.12	0.0867
SkinType	1	58.06	58.06	9.47	0.0043
Residuals	32	196.26	6.13		
Response Ds					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Flock	1	0.04	0.04	0.02	0.8861
SkinType	1	45.47	45.47	26.97	0.0000
Residuals	32	53.94	1.69		

” 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.

3.5 Wrinkle patterns over the body

The small *pin* wrinkles which all Merinos have do not seem to make any pattern. They are uniform across the body of the sheep. We are considering, in this section, patterns in the large folds which develop (in wrinkled sheep only) after birth and up to maturity. These form a very consistent pattern which was documented by Carter(1943) [3]. Dr Carter actually named each fold,

and recognized that each fold along the body of the sheep was associated with successive vertebrae along the spine. The pattern is therefore very regular from sheep to sheep. Only the size of folds varies, not the pattern.

Figure 11 shows a photograph of two Merino ewes, with and without wrinkle.



Figure 11: Two Merino ewes from Flock 1 of Trial 2, one wrinkled and one wrinkle-free

The wrinkled sheep in Figure 11 is a good example of the pattern to which we refer. Each fold runs vertically from dorsal to ventral positions, and there are approximately the same number of folds as vertebrae. So each fold appears to mark the position one *dermatome* area of skin, with the main nerve from the spine running either under the fold or between the folds. We do not know the spatial relationship between folds and nerve channels, but it appears to be a one-to-one relation.

The folds on the side of a sheep always run vertically. The rows of follicle groups on the side of a sheep always run vertically. In mosaic sheep (Fraser and Short(1960) [5]), which are somatic fleece mutations, the patterns of mutant fleece always run vertically. These phenomena reflect the way the skin tissue develops. The skin develops as a series of separate patches called dermatomes, each patch being associated with one nerve descending from the spine. It is not clear why fold development follows this pattern, but it is quite obvious that it does so.

4 Discussion

We have established the following from observations on adult Merino sheep

- wrinkled sheep have skin which is less supple and less compressible
- wrinkled sheep have more collagen in the lower dermis
- wrinkled sheep have more Type I collagen in the lower dermis
- in wrinkled sheep the collagen in the lower dermis extends upwards around the follicle bulbs into the upper dermis
- within wrinkled sheep, there is no difference in collagen between sites on a wrinkle or between wrinkles
- wrinkled sheep have more highly curved follicles
- wrinkled sheep have lower follicle density and lower S/P ratio as adults
- wrinkled sheep have coarser fibre diameters, both primary and secondary fibres as adults

In addition we know the following from other published work

- wrinkles can be observed forming in foetal skin of Karakul and Merino lambs at around 100 days of gestation (Bogolyubsky(1940 [1]))
- pin wrinkles are small and are present at birth and remain into adulthood. They are mainly a characteristic of Merino sheep (Carter(1943) [3])
- large folds grow in size as the sheep matures, but can be visible at birth. They are also mainly a characteristic of some Merino sheep. Large folds form in a pattern in which there is a one to one relation between folds and dermatomes (Carter(1943) [3])
- large folds consist of epidermis, papillary dermis, and lower or reticular dermis, but not the muscle and fat layers (Mitchell et al (1984) [22])
- collagen is present in the foetal dermis from about day 80, ie at about the same time as when the secondary derived follicles are forming (Knight et al (1993) [16])
- collagen in the dermis gradually becomes more Type I as the sheep matures (Knight et al (1993) [16])
- collagen in the dermis changes from a reticular arrangement to a complex arrangement with intertwining bundles of fibres, starting at about 5 months of age. (Kozlowski (1966) [17])
- developing follicles in the foetus can be seen to be curved, before they grow a fibre (Hardy and Lyne (1956) [7])

- in developing skin of Karakul sheep follicle bulbs can be seen to be deflected sideways by the collagen layer in the dermis (Dreyer, et al (1983) [4])

We have three hypotheses which may explain the above observations

4.1 Two layer folding hypothesis

Perhaps the most important result above is the negative one. There were no histological differences between skin sampled on a fold or between folds in wrinkled sheep. A fold is therefore not an additional organelle which grows on top of the skin; the tissues within a fold are exactly the same tissues as in skin between folds. What , therefore causes the skin to fold?

We maintain that skin folds are formed the same way as all other tissue folds. A fold forms because some layers of the skin grow faster than other layers. Any dual layer structure will curve if one layer changes length or area faster than the other layer. A bimetal strip is one example. In biology, curved surfaces are formed by non-allometric growth. (Thompson(1917) [30]).

We can identify the layers involved. In the case of large folds, we know from Mitchell et al (1984) [22] that a fold contains epidermis, papillary dermis and reticular or lower dermis, but not the muscle and fat layers. So the two super-layers that differ in growth rate are (a) layers 1,2, 3 together, and (b) layers 4 and 5 together. As sheep mature and large folds form super-layer (a) grows faster. So why do sheep with large folds have more collagen and more Type 1 (hard) collagen? Because the presence of hard collagen in the lower dermis binds the lower edge of the dermis to the muscle and fat layers below and prevents them from expanding to match the growth of super-layer (a). The collagen binds the boundary between super-layers (a) and (b).

That is not the whole story of large folds. For large folds to form there has to also be excessive growth of super-layer(a) as the sheep matures. We are not sure whether this excess growth of (a) occurs as a result of maturation of the large number of secondary original follicles in Merinos, or whether it is just a genetic signal to grow more skin. What happens in Merino sheep without large folds is that the excess growth of super-layer (a) still occurs, but super-layer (b) is not bound by hard collagen at the boundary with super-layer(a), so both layers can expand together and the skin on the sheep feels loose and supple. Other breeds of sheep (eg British breeds) do not have the excessive growth of super-layer (a) as they mature, so they do not form folds, regardless of whether they have hard collagen. This suggests that the excessive growth of super-layer (a) in Merinos is indeed due to the need to fully develop large numbers of secondary original follicles and their accessory glands.

In the case of pin-wrinkle, we know that the tiny pin-wrinkle folds start to form in-utero at around days 80 to 100. That is exactly the time window in which the large population of secondary original follicles is forming in Merino sheep. We suggest that the formation of large numbers of secondary original follicles dramatically increases expansion of the epidermis and papillary dermis, while the lower dermis is held at a slower growth by the development of collagen.

So in the case of pin-wrinkle the super layers are (a) epidermis and papillary dermis, and (b) lower dermis, muscle and fat layers.

So the difference between large folds and pin wrinkle is in the layers involved and in the time of formation. The two layer hypothesis applies to both.

4.1.1 Two factor wrinkle formation hypothesis

Given the above, we are led to suggest that there are two independent factors involved in fold formation

presence of hard collagen in lower dermis this prevents the hypodermis from expanding to match upper dermal growth

secondary original follicles this leads to excessive growth of the upper dermis both in utero and as the sheep matures.

These 2 factors apply to both pin wrinkle and large folds. It is just the layers that differ.

4.2 Follicle curvature hypothesis

Perhaps the next most important result is the observation that wrinkled Merinos invariably have curved follicles. We suggest that this happens because the downgrowth of follicle plugs from the epidermis into the papillary dermis which occurs from days 60-100 in the foetus is interfered with by the presence of large amounts of developing collagen in the lower part of the papillary dermis and the reticular dermis. Growth of follicle plugs downward is inhibited by the presence of collagen and they continue to grow, but deflect sideways.

There are photomicrographs in Hardy and Lyne (1956) [7] in which one can actually see follicle plugs curved, before the are growing a fibre. If this is correct we have actually solved the 'chicken and egg' argument about fibre curvature and follicle curvature. Follicle curvature comes first. The fibres curve because they grow into a curved tube. The bilateral ortho and para cortex arrangement in curved Merino fibres is a consequence of the fibre developing in a curved tubular space, not a cause of the curvature. The cortical cells differentiate differently on the inside of the curve because they are in a more cramped space.

So the spatial distribution of collagen is involved in follicle curvature. That is not quite the same thing as presence of hard collagen, but it suggests that follicle curvature and wrinkle have one common cause.

4.3 Auxiliary issues

The pattern of folds over the sheep's body noted in section 3.5 is not fully understood. The fact that the folds always run in the same direction implies that either the expansion in superlayer (a) is directional, or the collagen binding in super-layer (b) is directional, or some other factor interferes to provide a direction. We are not sure, but we favour the last possibility, because another

factor can be identified. We have noted that each fold occupies one dermatome. A dermatome is an area of skin associated with one major nerve channel which runs from the spine downwards. The position of the nerve channel may be involved in deciding where the skin is to fold. The major nerve channel is in the hypodermis, and minor nerves run from there into the dermis, like risers in a plumbing system. So at the position where the 'risers' cross from hypodermis to dermis the two layers cannot move independently. So at these points the two layers would be 'anchored' together. There are rows of such 'anchor points' running from the spine downward. The skin folds parallel to these rows. We do not know if the rows of anchor points are under the folds, or between folds. That is, we do not know where the major nerve channel is in relation to the fold. One might guess that the 'anchor points' are between folds so that the expansion of super-layer (a) between each pair of anchor points forms the fold.

There are also major blood vessels in the hypodermis, both arteries and veins. These also have minor branches which cross the boundary into the dermis, like risers. There is some information on nerves and blood vessels in sheep skin in Lyne and Hollis (1968) [19], but we have been unable to find the exact arrangement of blood vessels. The same considerations apply as for nerves, the blood vessels may determine 'anchor points' at which the dermis cannot move against the hypodermis.

Wrinkled and wrinkle-free sheep also differ in follicle density, S/P ratio, diameter of primary fibres (D_p) and diameter of secondary fibres (D_s). Three of these (follicle density, D_p, and D_s) can be understood as effects of the greater expansion of the upper skin layers in wrinkled sheep. Wrinkled sheep have more skin area, but they do not have more follicles per head, because the follicles are all formed in utero, while the skin expansion occurs after birth as the sheep matures. This simply spreads the follicles over more skin, and the density is less. The effects on D_p and D_s are a consequence of the density effect. However the lower S/P ratio of wrinkled sheep is not thus explained. The diameter results are confirmed by Scobie et al (2005) [28].

Apart from being more curved, follicles in wrinkled sheep are also longer. Both the straight length and the curved length are greater. Also the traditional follicle depth measure, which is the average vertical distance of follicle bulbs below the skin surface, is also larger in wrinkled sheep. If the presence of collagen makes developing follicles curve, is it possible that it also prolongs their development so that they grow longer?

There is a biological connection between the development of follicles and the development of collagen. The papilla cells in follicles are differentiated fibroblasts. The fibrocyte cells which produce collagen fibres are also differentiated fibroblasts. There is an established theory about the way pre-papilla cells distribute to follicle papillae, and the effects this has on follicle density and fibre diameters (Moore et al (1989) [23], Moore et al (1998) [24]). There is no such theory, that we are aware of, for collagen. It may be that the population of fibroblast cells is limited in number at some stages so that a *tradeoff* situation might exist between follicle development and collagen development. This might explain the lower S/P ratio of wrinkled sheep.

4.4 Predictions and verifications of hypotheses

We can check if the above hypotheses are robust by using them to predict some previously unobserved phenomena. We make a number of predictions and check each in turn against new data.

4.4.1 Wrinkle epistasis

The two factor wrinkle formation hypothesis asserts that for skin folds to form there must be both hard collagen binding the lower dermis, and excessive growth of the upper dermis probably attributable to large numbers of secondary original follicles. Under this model, only sheep with both factors present at a sufficient level will form folds. This implies that the two factors interact. So we predict that the quantitative genetics of wrinkle will involve an epistatic interaction between the genes for hard collagen and the genes for large number of secondary follicles. This is something which can be checked.

We were able to access data from five CSIRO experimental flocks in which degree of wrinkle had been observed according to the photographic standards of Turner, et al (1953). These flocks were fully pedigreed and contained a total of 22200 sheep with data. A mixed model was fitted which removed fixed effects and estimated components of variance of wrinkle score for individual environment, individual additive genetic, individual additive x additive epistatic, maternal additive genetic, and maternal environmental components, for each flock. Here we just present a summary as a pie chart in Figure 12 showing the average of the component estimates over the five flocks, as percentages of total variance.

There is a full writeup of these analyses available in Jackson and Watts (2018) [11]. These analyses are too extensive to present here. The conclusion, however, is important. Figure 12 shows that 29 percent of the variance of wrinkle is additive genetic, and 18 percent is additive x additive epistatic. We regard this as a verification of the two factor hypothesis.

4.4.2 Follicle orientation

The follicle curvature hypothesis asserts that presence of large amounts of collagen in the developing foetus interferes with downgrowth of the follicle plugs causing them to curve. If this is correct one would also expect

- fibres from such follicles to have more variable emergence angles,
- the plane of curvature of follicles and fibres to be more variable
- follicle groups to be more disrupted

We were not able to check on all of these predictions, but there is a small amount of data shown in Table 11 comparing wrinkled and wrinkle-free sheep for fibre emergence angle and follicle group orientation.

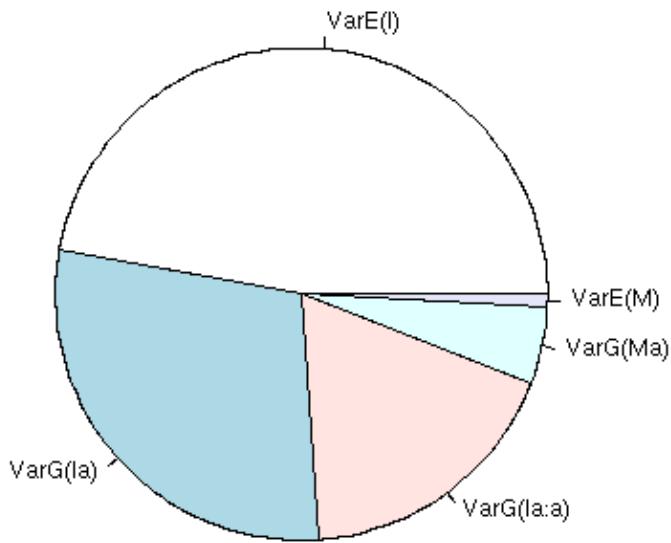


Figure 12: Summary of analyses of quantitative genetic variation in wrinkle score. The piechart shows percentages of variation attributed to the following variance components: VarE(I) = individual environmental variance, VarG(Ia) = individual additive genetic variance, VarG(Ia:a) = individual additive x additive epistatic variance, VarG(Ma) = maternal additive genetic variance, and VarE(m) - maternal environmental variance. The variance components are averages of estimates for five Merino flocks.

The wrinkled sheep are certainly more variable in bot fibre emergence angle and follicle group orientation. We have no data on plane of curvature. That would involve looking at the orientation of the bilateral ortho/para cortex segmentation.

It is clear that the follicles in wrinkled sheep are more dislocated, both singly and in groups. That does not prove that the foetal collagen development has caused this. We can get closer to a verification by plotting amount of collagen

Table 11: Means for coefficient of variation of fibre emergence angle and coefficient of variation of follicle group orientation, for wrinkled and wrinkle-free sheep from both flocks of Trial 2

Flock	Skin.type	Fibre emergence angle CV (%)	Follicle group orientation CV (%)
1	wrinkle-free	14.3	13.3
	wrinkle-free	16.2	15.2
2	wrinkled	24.0	29.2
	wrinkled	25.0	37.2

(measured by integrated optical density of PSR stained sections) in adult skin sections against CVof fibre emergence angle for individual sheep. This is done in Figure 13

We see a correlation of 0.47 which is significant.

4.4.3 Curvature and diameter

4.4.4 Non-Merino breeds

4.4.5 Pin wrinkle and secondary original follicles

5 Conclusion

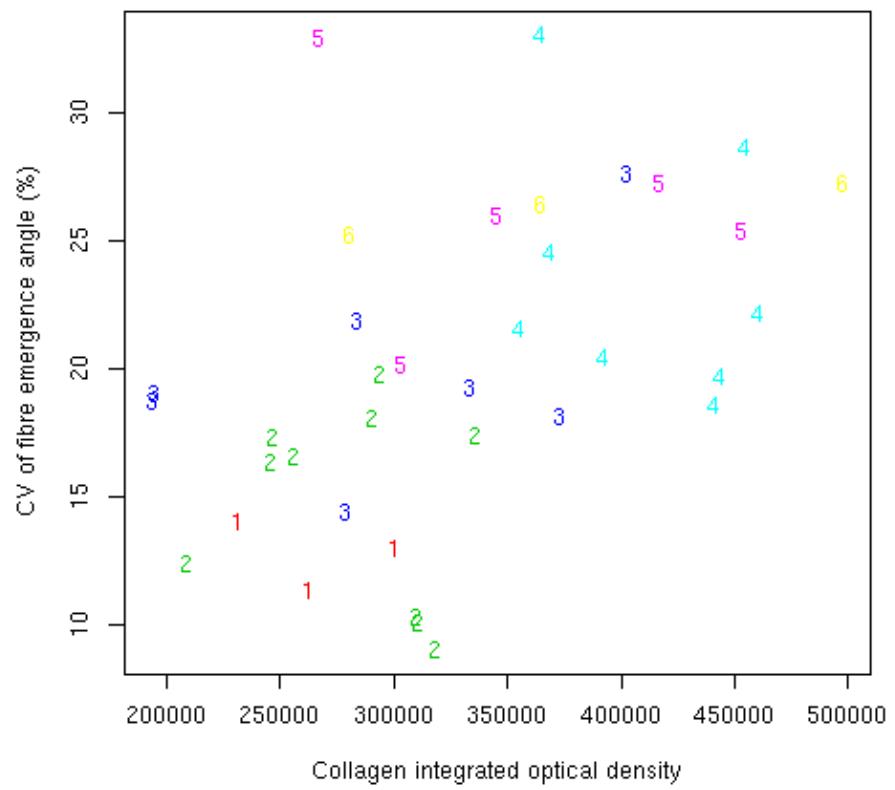


Figure 13: Plot of amount of collagen in adult skin against coefficient of variation of fibre emergence angle for individual sheep from Trial 2. The numbered points are follicle curvature scores

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