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2 **Histology of collagen in Merino sheep skin and its association with**  
3 **skin wrinkle formation**

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7 **ARTICLE HISTORY**

8 Compiled October 5, 2020

9 **ABSTRACT**

10 Skin of Merino sheep contains collagen in the lower dermis. Amount and type of  
11 collagen (Type I or Type III) are shown to be associated with formation of skin  
12 wrinkles. It is proposed that wrinkles form when papillary dermis grows faster than  
13 the sub-dermis and the two are firmly bound together by presence of Type I collagen.  
14 It is also proposed that the large number of secondary follicles in Merino sheep is  
15 implicated in overgrowth of the papillary dermis. Skin wrinkles will only form in  
16 the presence of these two interacting factors. Consequences for breeding low-wrinkle  
17 Merinos are explored.

18 **KEYWORDS**

19 Sheep; skin; collagen; wrinkle; fold

20 **1. Introduction**

21 This study is an attempt to understand the histological structure of skin wrinkles in  
22 Merino sheep, and the process of their formation. The basic biology of Merino skin  
23 wrinkles needs to be examined, as an essential preliminary to research into methods  
24 that may remove wrinkles, whether by breeding or direct intervention.

25 There have been few attempts to define what a sheep skin wrinkle actually is. Early  
26 work of (Carter 1943) went as far as describing and naming all wrinkles on the neck,  
27 body, and breech of Merinos, and developed a set of photographic scores for degree of  
28 wrinkle. Carter used the terms *fold* and *wrinkle* interchangeably, noting that common  
29 usage was for *fold* to refer to larger wrinkles, but he distinguished small *pin wrinkles*  
30 present in all Merinos, from larger wrinkles which develop to various degrees as a sheep  
31 matures. From this early start, somewhat surprisingly, nothing on biology of wrinkles  
32 appears until the study of (Mitchell et al. 1984).

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

34 **Layer1** epidermis is mainly keratinised protein

35 **Layer2** contains wool follicles and accessory glands and is part of the dermis. Some-  
36 times called *papillary dermis*.

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

<sup>39</sup> reticular, but may be interwoven.

<sup>40</sup> **Layer4** contains voluntary muscle, collagen and elastin

<sup>41</sup> **Layer5** adipose tissue

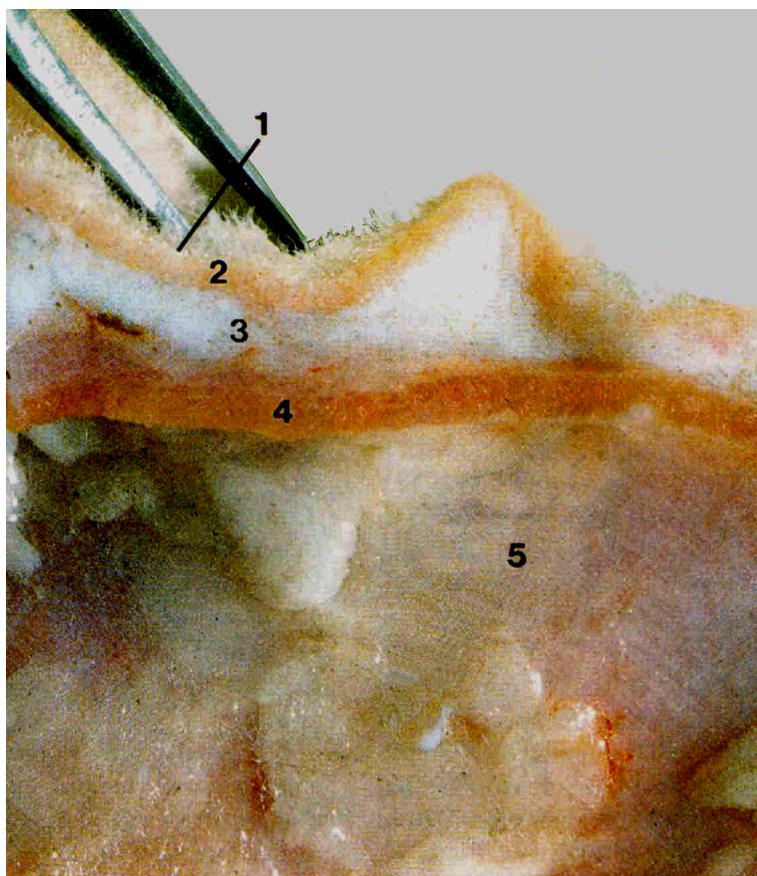


Figure 1.: Merino sheep skin showing tissue layers. 1. epidermis with wool fibres; 2. papillary layer of dermis; 3. reticular layer of dermis; 4. areolar tissue and muscle; and 5. adipose tissue. One wrinkle is present on the right-hand side of the forceps. Forceps opening is 5mm. Modified from (Mitchell et al. 1984).

<sup>42</sup> Only the first 2 layers curve upward in a folded section of skin, layer 3 expands  
<sup>43</sup> to fill space under the wrinkle, layers 4 and 5 remain straight. This may be seen in  
<sup>44</sup> Figure 1. Mitchell et al. noted that Layer2 is more elastic than Layer 3. It appears  
<sup>45</sup> as if wrinkles are formed either by an overgrowth of Layers 1-2, or by a shrinkage  
<sup>46</sup> or tightening of Layer 4. Mitchell has demonstrated that if Layer4 (and Layer 5) are  
<sup>47</sup> dissected away from a skin specimen with wrinkles, the folds in Layers 1-2 flatten.  
<sup>48</sup> Therefore in a wrinkled sheep, Layer 4 is holding the skin under some tension, which  
<sup>49</sup> relaxes when Layer 4 is removed.

<sup>50</sup> Wrinkle development has been even less studied. Merino lambs are born with vis-  
<sup>51</sup>ible wrinkles. (Bogolyubsky 1940) asserted that wrinkles were observed forming in  
<sup>52</sup> foetal skin of Karakul and Merino lambs at around 100 days of gestation, which is  
<sup>53</sup> about the time at which secondary derived follicles initiate (Fraser and Short 1960).  
<sup>54</sup> A photograph of skin surface of a 10-day old Merino lamb (Carter 1943, see) shows

55 fine wrinkles of the type Carter termed *pin wrinkles*. Whilst studies of follicle development are extensive (Fraser and Short 1960; Ryder and Stevenson 1968; Maddocks and Jackson 1988, see), similar studies of foetal wrinkle development are lacking.

56 To bring new information to bear on wrinkle formation, this study focusses on  
57 amount, type, and arrangement of collagen in skin. Collagen is found in the dermis  
58 (layers 2 and 3) of foetal skin at the time follicles develop (Knight et al. 1993). Knight  
59 et al. distinguish two collagen types (Type I or 'hard' collagen and Type III or 'soft'  
60 collagen) and note that Type III is most prevalent at 75 days of gestation, and its  
61 proportion falls progressively as the foetus develops. Type I is least prevalent at day  
62 75 and its proportion rises to over 50 percent by birth.

63 In histological examination of skin, Type I or hard collagen forms thick bundles of  
64 eosin staining fibres. Its function is to bind tissues together in a rigid manner. Type III  
65 or soft or reticular collagen forms thin separate eosin staining fibres which cross-link to  
66 form a fine flexible mesh network supporting soft tissues. The strength, elasticity and  
67 flexibility of skin comes from presence of collagen and elastin fibres, and presumably  
68 variations in these properties derive from variations in amounts and proportions of  
69 these types of collagen. The basis of this study is an hypothesis that amount and type  
70 of collagen in the lower dermis determines how well the upper dermis and sub-dermis  
71 are bound together, and hence the likelihood that skin will form wrinkles.

72 Collagen fibres are formed by fibroblast cells. At 75-80 days fibroblasts appear as  
73 round, immature cells (Knight et al. 1993) surrounded by reticular collagen fibres which  
74 are composed of Type III collagen and form a net-like structure. By birth fibroblasts  
75 have matured and collagen fibres can be inter-meshed to various degrees forming thick  
76 bundles of fibres which are birefringent. If the fine reticular or net-like fibre pattern  
77 remains, the mature sheep has soft or Type III collagen; if fibres inter-mesh and form  
78 thicker and longer bundles the mature sheep has hard or Type I collagen.

79 Collagen development, secondary follicle development and wrinkle formation all  
80 seem to commence at the same time of 75-100 days of foetal age. Follicle initiation  
81 ceases at birth ( 150 days) but development of collagen and wrinkles continues into  
82 maturity. In this study we look at end point of development - that is we study collagen  
83 and follicles in adult sheep with and without wrinkles.

## 86 2. Materials and Methods

87 The experimental design was to choose, by visual inspection, individual sheep with  
88 wrinkle-free skin and wrinkly skin from each of several Australian Merino flocks.

89 Two trials were conducted

90 **Trial 1** Two sheep were chosen from each of six SRS® Merino stud flocks, one wrinkle-  
91 free and one wrinkled. This is a randomised block design without replication .  
92 The blocks are the flocks, and the treatment is presence or absence of wrinkle.

93 **Trial 2** Eighteen sheep were chosen from each of two commercial flocks, nine wrinkle-  
94 free and nine with wrinkles. This is a randomised block design with replication.  
95 The second of these two flocks was more wrinkled.

### 96 2.1. Skin samples

97 In Trial 1 a biopsy sample was taken from the mid-side position on each sheep and  
98 specimens were trimmed (Maddocks and Jackson 1988) before processing, so that only

99 Layers 1-3 were present for histological observation.

100 In Trial 2 , for the sheep with wrinkled skins, skin biopsies were collected from  
101 on-wrinkle as well as off-wrinkle positions. For the wrinkle-free sheep only one biopsy  
102 sample was collected. These specimens were not trimmed, so they included Layers 1-3,  
103 and in some cases part of Layers 4 and 5, depending on the depth of biopsy.

104 Mid-side skin biopsy samples were collected using a 10-millimetre circular trephine  
105 (Acu Punch skin biopsy punches, Acuderm, Inc.) and fixed in 10% buffered formol  
106 saline solution.

## 107 ***2.2. Histological skin processing and observations***

### 108 ***2.2.1. Collagen observations***

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

116 Sections were then reviewed microscopically (BX53 Olympus, Australia)), and im-  
117 ages taken on 3 CCD camera (DP72, Olympus, Australia) under both bright field and  
118 polarised conditions.

119 For PSR collagen analysis, a 40x objective was employed at a fixed exposure to take  
120 high power images of 5 random lower dermal fields of view for image analysis aimed  
121 at determining amount of collagen in each field.

122 The five images for each sample were then uploaded for quantitative analysis via the  
123 ImagePro Plus (Media Cybernetics, USA) 7.1 software in which thresholds were set  
124 to count all pixels comprising of the red staining fibres in the PSR stained specimen  
125 field. This provided a measure of area of the field occupied by red stained collagen  
126 fibres.

127 A measure of total amount of collagen in the field could be obtained by allowing  
128 for the intensity of red staining of each pixel. This is a measure density of collagen  
129 within the pixel and depth of collagen through the thickness of the section. Grey-  
130 values for each pixel were converted to optical density, and optical densities summed  
131 (ie integrated) over all pixels in the field. Means were calculated for each specimen,  
132 averaged over 5 fields, and graphed. Optical density data for each field subjected to  
133 analysis of variance to test for differences between wrinkle-free and wrinkled sheep,  
134 and , in Trial 2, to test for differences between on-wrinkle and off-wrinkle specimens  
135 within wrinkled sheep. All specimens were measured in this way, and this is the main  
136 quantitative result of the study.

137 Polarised light was employed to determine type of collagen present within each  
138 sample. Bundles of fibrils stained with Sirius Red dye are strongly birefringent; single  
139 fibrils as in reticular collagen are not (Cuttle et al. 2005). Collagen stained with PSR  
140 has enhanced birefringence compared with that in H-E stained sections (Junqueira  
141 et al. 1979). Under polarised light sections show coloured red,orange, yellow, or green,  
142 in order of thickness of bundles of fibres. Thus red or orange should indicate Type I or  
143 hard collagen (which has thick bundles of fibres) while yellow or green should indicate  
144 Type III collagen which has individual fibres in a net-like structure.

145 Attempts to use polarised light images to make quantitative assessments of amounts

146 of each Type of collagen have been criticised (Lattouf et al. 2014). The main issue  
147 seems to be that birefringence is directional, only fibres aligned with the direction of  
148 polarisation will show colours. We refrained from attempting this quantification, so  
149 our polarised light results are only qualitative.

150 **2.3. Statistical Methods**

151 Data were imported into the R statistical program (R Core Team 2017) and analysed  
152 using the *aov()* function for analysis of variance. Allowance was made for sub-sampling  
153 design by choosing an appropriate error level for F tests in analysis of variance.

154 **3. Results**

155 We look first at overall morphology of skin specimens, then at details of collagen  
156 structure, and finally at related observations

157 **3.1. Skin tissue Morphology**

158 Pairs of wrinkle-free and wrinkled sheep from each flock in Trials 1 and 2 showed  
159 consistent visual differences in their tissue structure. Figure 2 shows vertical sections  
160 stained with H-E from a wrinkled and wrinkle-free pair of sheep.

161 The connective tissue of the lower dermis below follicle bulbs was more heavily  
162 stained in wrinkled sheep. The stained lower dermal material is in clumps in wrinkled  
163 sheep, whereas in wrinkle-free sheep the proximal connective tissue has a finer more  
164 uniform structure. These differences were consistent across all sheep.

165 Although the Trial 2 biopsy samples were not trimmed before sectioning, the spec-  
166 imens displayed in Figure 2 do not show any layers below Layer 3. This is because  
167 biopsy specimens are not regularly taken deep enough to include layers 4 and 5.

168 To check if connective tissue extends into Layers 4 and 5 we look at a deeper biopsy  
169 specimen that has Layer 4 intact. Figure 3 shows one example section which is from  
170 a wrinkled sheep.

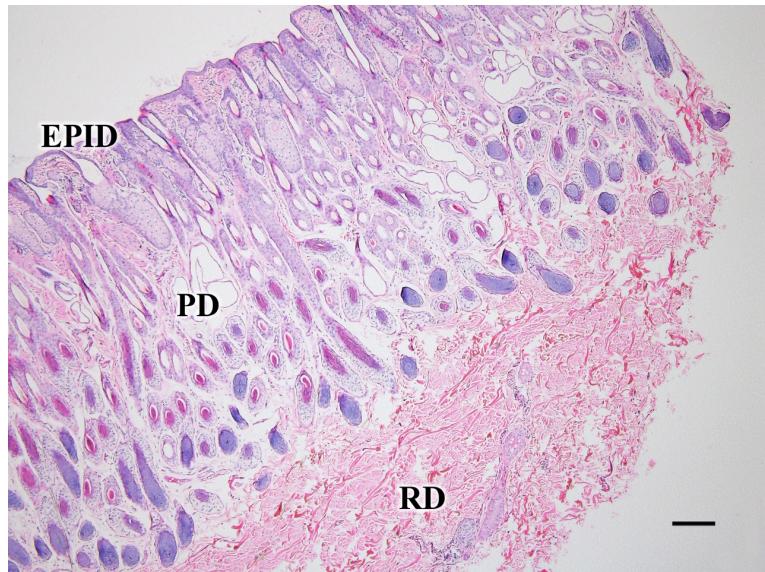
171 Figure 3 shows connective tissue in Layer 3 (lower or reticular dermis), followed by a  
172 thin layer of adipose tissue , then a wider layer of muscle tissue (stained pink with eosin)  
173 evidently bordered by thin bands of connective tissue, which has a denser appearance  
174 compared to connective tissue in the reticular dermis. A trace of adipose tissue is  
175 present below the muscle layer,as in Figure /reffig:mitchell; the biopsy specimen was  
176 not taken deep enough to include all of Layer 5.

177 Our focus is on connective tissue in the lower dermis. We wish to quantify and  
178 qualify the way in which it differs between wrinkled and wrinkle-free sheep.

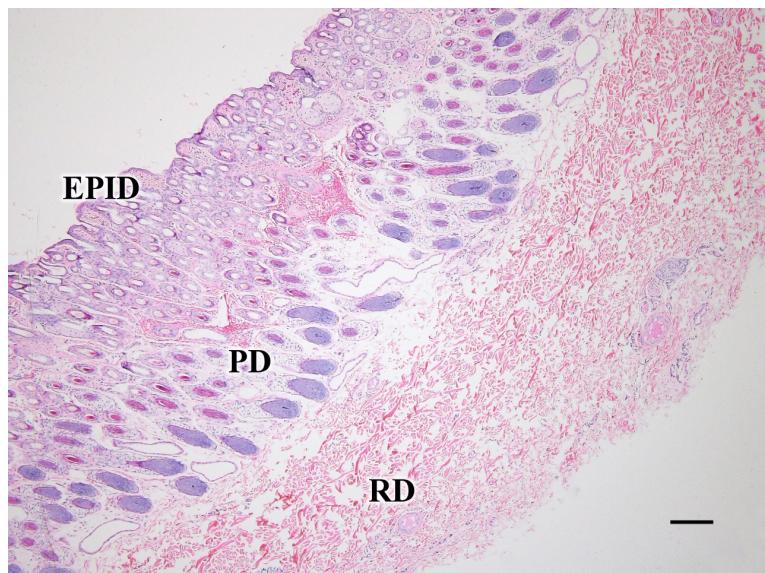
179 **3.2. Detailed morphology of connective tissue**

180 The stain picosirius red (PSR) differentiates collagen from other components of con-  
181 nective tissue. Figure 4 shows a section from the same sheep as Figure 3 stained with  
182 PSR and examined with bright field microscopy.

183 Collagen is stained red. Some collagen is present in Layer 2 (papillary dermis), a  
184 dense band of collagen occurs in Layer 3 (sub-papillary dermis), and two narrow bands  
185 of very dense collagen are present either side of the muscle tissue which is stained yellow



(a) Sheep 3437 Wrinkled



(b) Sheep 3457 Wrinkle-free

Figure 2.: Vertical sections from a wrinkled (a) and a wrinkle-free (b) sheep from Trial 2 flock 1 stained with H-E. Skin layers are: **EPID** epidermis, **PD** papillary dermis, and **RD** reticular dermis. Scale bar is 200  $\mu\text{m}$

186 by the PSR stain. Wool fibres and follicle bulbs are stained yellow by the picric acid  
 187 component of PSR. Within the muscle tissue are tiny tracks of red stained connective  
 188 tissue. The connective tissue in layer 4 is separated from that in Layer 3 by a thin band  
 189 of adipose tissue and appears to have a different structure. Our focus is on connective  
 190 tissue in the reticular dermis, because this tissue determines how strongly the upper  
 191 dermis is bound to the hypodermis.

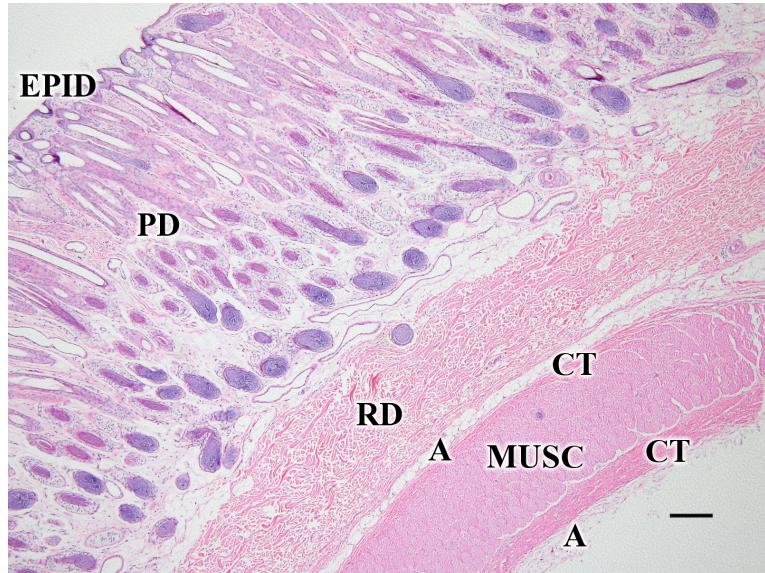


Figure 3.: Vertical section from a wrinkled sheep (3456) from Trial 2 Flock 1 stained with H-E. This section is from an untrimmed biopsy specimen and shows all 5 layers identified by Mitchell: **EPID** epidermis, **PD** papillary dermis, **RD** reticular dermis, **MUSC** muscle, and **A** adipose tissue). In addition there are two layers of **CT** connective tissue, either side of the muscle layer, and a thin layer of **A** adipose tissue between the reticular dermis and the muscle. Scale bar is  $200\mu m$ .

192    3.2.1. *Amount of collagen*

193    Since the nature of the connective tissue in Layer 3 is what seems to differ between  
194    wrinkled and wrinkle-free sheep, we attempt to quantify it.

195    To quantify collagen in Layer 3, five fields under a 40x objective were chosen at  
196    random from within Layer 3 of each PSR stained section from each sheep. A typical  
197    image from one field of a wrinkled and a wrinkle-free sheep is shown in Figure 5.

198    The two fields shown in Figure 5 illustrate the difference between wrinkled and  
199    wrinkle-free sheep. They show that collagen in Layer 3 of wrinkled sheep is in larger  
200    (thicker and longer) aggregates (bundles of collagen fibres) and collagen within each  
201    bundle is more dense. So the collagen bundles in Figure 5(a) take up considerably more  
202    3 dimensional space than those on Figure 5(b). More collagen is therefore present in  
203    wrinkled sheep. This was confirmed with quantitative data.

204    Image analysis was used to assess total amount of red stained pixels in each field.  
205    The sum of calibrated optical densities of all pixels in the red image was calculated.  
206    Integrated optical density for each sheep is shown in Figure 6 for Trial 1 and Figure 7  
207    for Trial 2.

208    These data are a measure of total amount of collagen tissue present in the microscope  
209    section at the position of the chosen field in the lower dermis. Total number of pixels  
210    in an image taken with a 40x objective was 1920000, so one could scale these optical  
211    density sums to average optical density of a pixel by dividing by 1920000. We can see  
212    that for Trial 1 wrinkle-free sheep always had less collagen, except for those in Flock

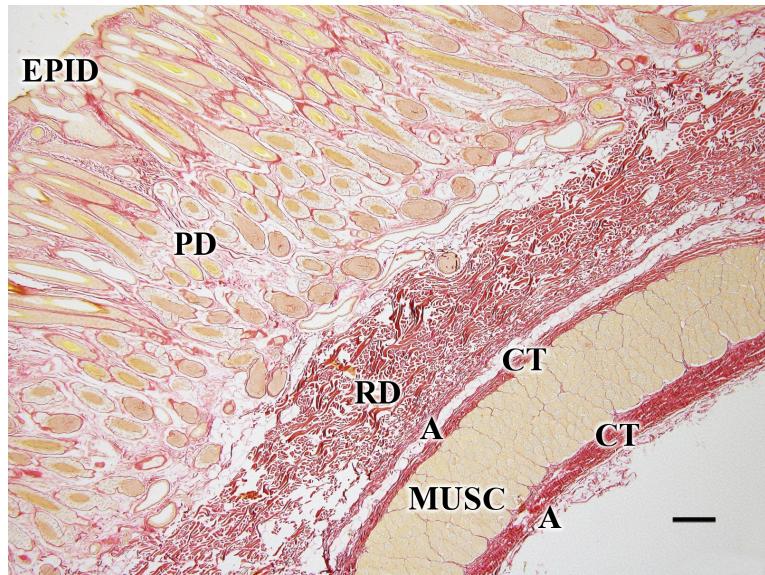


Figure 4.: Vertical section from a wrinkled sheep (3456) from Trial 2 Flock 1 stained with PSR and viewed with bright field microscope. This section is from an untrimmed biopsy specimen and shows all 5 layers identified by Mitchell: **EPID** epidermis, **PD** papillary dermis, **RD** reticular dermis, **MUSC** muscle, and **A** adipose tissue). In addition there are two layers of **CT** connective tissue, either side of the muscle layer, and a thin layer of **A** adipose tissue between the reticular dermis and the muscle. Scale bar is  $200\mu m$ .

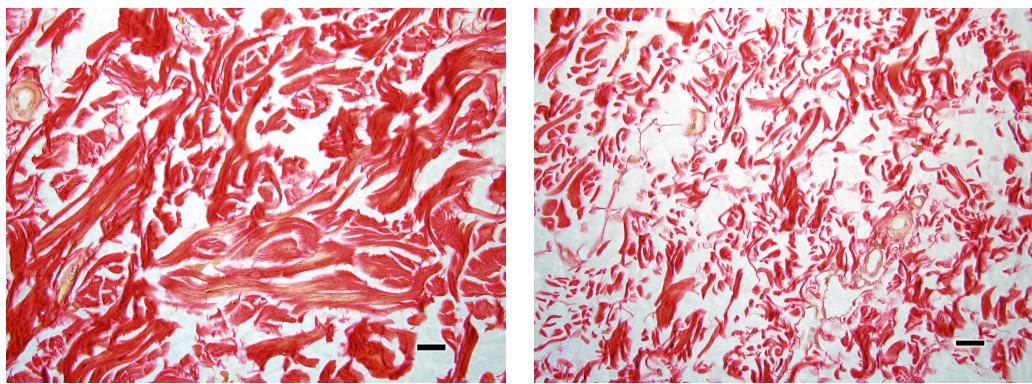


Figure 5.: Fields chosen at random from within Layer 3 (subpapillary dermis) of a wrinkled (a) and a wrinkle-free (b) sheep. Illustrates difference in collagen amount and structure. Stained with PSR and viewed with a 40x objective. Scale bar is  $20\mu m$ .

- 213 4. In Trial 2 wrinkle-free sheep always had less collagen than the off-wrinkle sample  
 214 from wrinkled sheep, but the on-wrinkle sample was more variable.  
 215 Significance of differences apparent in Figure 6 was tested by analysis of variance  
 216 extracting terms for FlockNo, SkinType, and their interaction, as presented in Table 1.

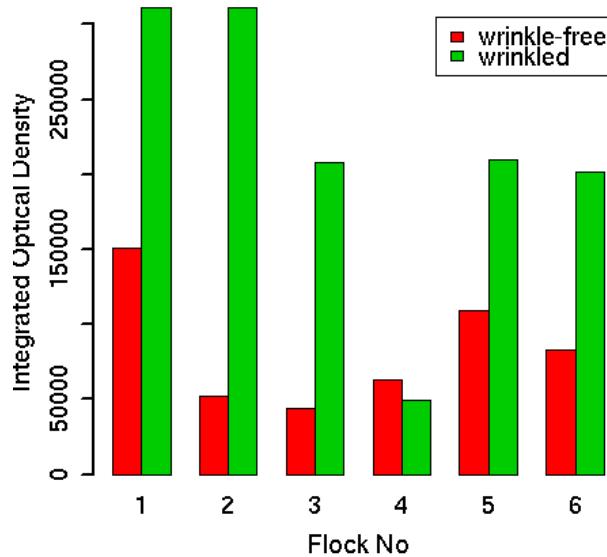


Figure 6.: Integrated optical density of the red images of sections stained with PSR for each sheep in Trial 1 averaged over five microscope fields

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

Source	Df	Mean Square x 10 <sup>8</sup>	F value	Significance
FlockNo	5	342.66	1.688	NS
SkinType	1	2593.20	12.775	*
FlockNo:SkinType	5	202.98	12.934	***
Residuals	48	15.69		

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

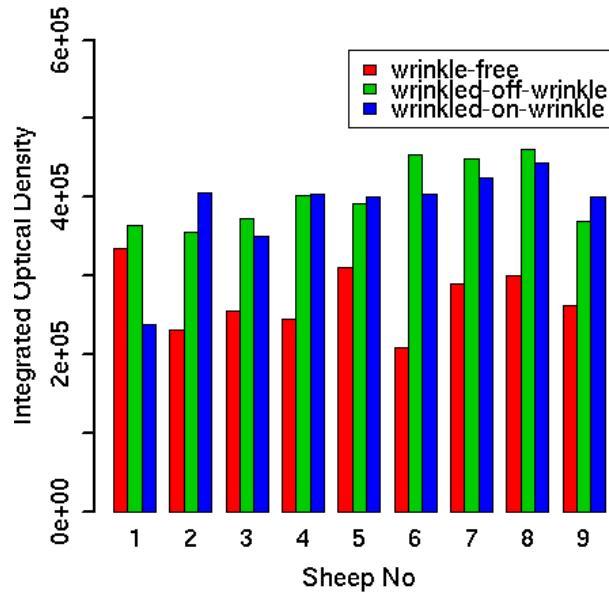
217

218 The residual term in Table 1 is variation between randomly chosen Fields within a  
219 specimen, because there were no replicate sheep within each Flock:SkinType subclass.  
220 The difference between wrinkled and wrinkle-free SkinTypes is significant at 5% level.  
221 Flock differences are not significant. An interaction was significant.

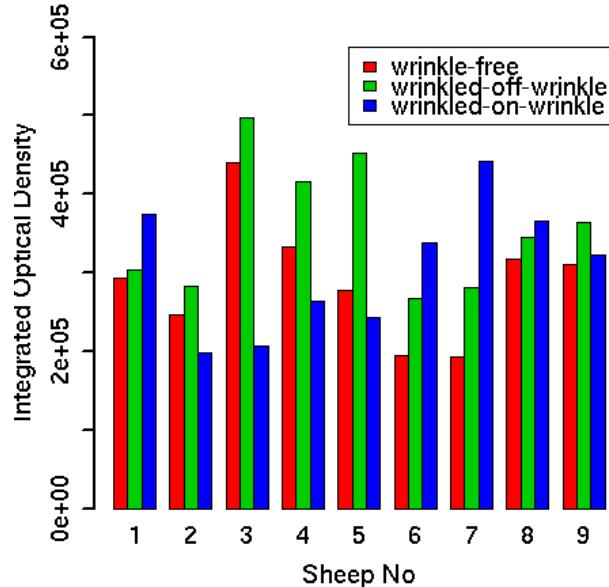
222 The equivalent analysis of variance for Trial 2 (Figure 7) data is shown in Table 2.

223 Differences between wrinkled and wrinkle-free SkinTypes are now shown to be highly  
224 significant. Flock differences were not significant and a significant interaction of Flock  
225 with SkinType was found.

226 The on-wrinkle and off-wrinkle sampling positions within wrinkled specimens were  
227 not significantly different. On-wrinkle specimens actually had a lower integrated optical  
228 density than off-wrinkle specimens indicating slightly less collagen on a wrinkle than  
229 between wrinkles. There was also a significant amount of variation between sheep



(a) Flock No 1 of Trial 2



(b) 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

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 2 represents.

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

Source	Df	Mean Square x 10 <sup>8</sup>	F value	Significance
FlockNo	1	915.48	4.14	NS
SkinType	1	4123.38	18.67	***
SampPos	1	503.25	2.28	NS
FlockNo:SkinType	1	1016.76	4.60	*
FlockNo:SkinType:SheepNo	49	220.76	9.02	***
Residuals	218	24.47		

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

232 In this analysis the Sheep term is the error term for all terms above it in the analysis  
 233 of variance table, whereas Trial 1 had no sheep replication and we were forced to use  
 234 the FlockNo:SkinType term as error. This explains why SkinType differences were less  
 235 significant in Trial 1.

236 Means and standard deviations for integrated optical density for both Trial 1 and  
 237 Trial 2 are shown in Table 3

Table 3.: Means and standard deviations for integrated red pixel optical density of wrinkled and wrinkle-free sheep in Trial 1 and Trial 2

Trial	Parameter	Wrinkle-free	Wrinkled (off-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

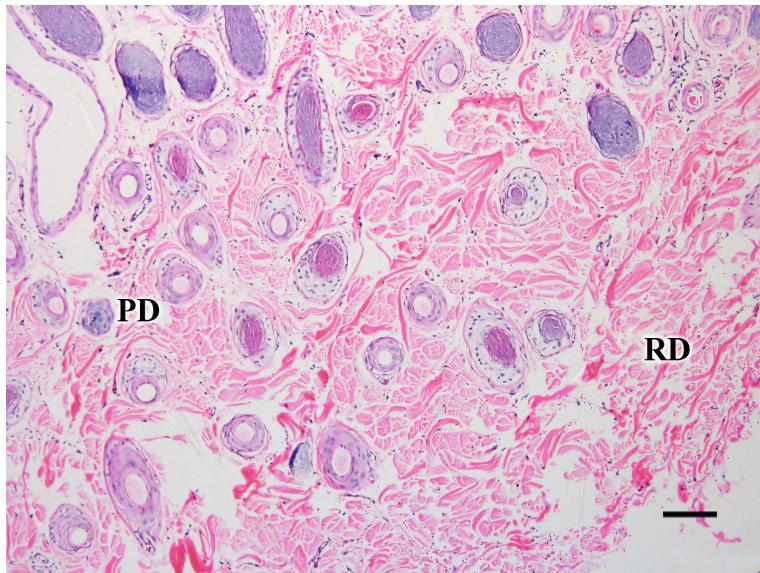
238 We see that wrinkle-free sheep actually have quite a low amount of collagen in  
 239 Trial 1. The Trial 2 sheep were from commercial flocks, and were generally more  
 240 wrinkled than those of Trial 1. Trial 2 wrinkled sheep ( either on-wrinkle or off-wrinkle  
 241 specimens) had a higher amount of collagen than wrinkled sheep from Trial 1.

242 The data and analyses show that more collagen is present in the lower dermis of  
 243 wrinkled sheep than wrinkle-free sheep. The actual size of the difference varied from  
 244 2.5 x in Trial 1 to 1.4 x in Trial 2. Within wrinkled sheep we detected no difference in  
 245 amount of collagen between samples taken on a wrinkle or in-between wrinkles.

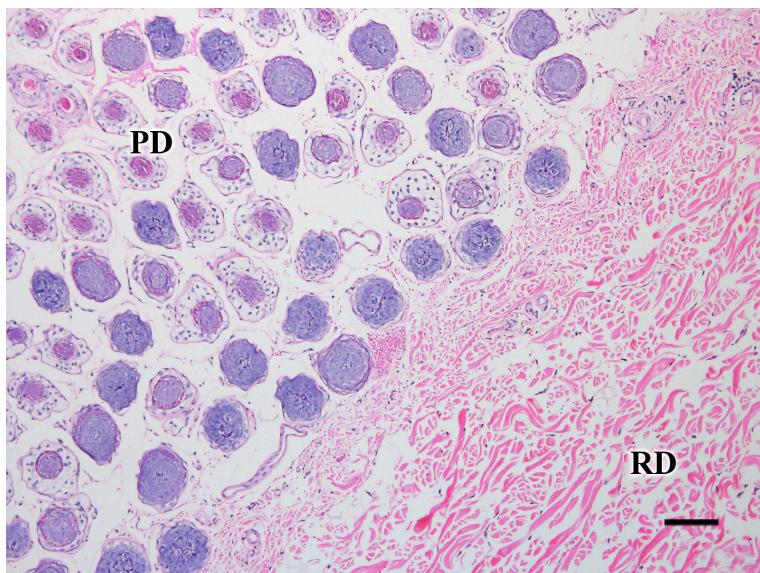
### 246 3.2.2. Spatial location and structure of collagen

247 It has been established that wrinkled sheep have more collagen . We now investigate  
 248 location of collagen in the dermis and whether arrangement of collagen fibres varies.

249 Figure 8 shows images of layers 2 and 3 in specimens from two sheep, one being  
250 wrinkled (a off-wrinkle specimen) and one being wrinkle-free.



(a) Sheep 3453 Wrinkled



(b) Sheep 3458 Wrinkle-free

Figure 8.: Vertical sections from a wrinkled (a) and a wrinkle-free (b) sheep from Trial 2 flock 1 stained with H-E, and viewed with a 10x objective. Skin layers are: **PD** papillary dermis, and **RD** reticular dermis. Scale bar is 80 $\mu$ m.

251 In the wrinkled sheep specimen collagen extends up into the follicular region, there  
252 being conspicuous amounts of collagen in and around follicle bulbs. In the wrinkle-free  
253 sheep there is little collagen in amongst follicle bulbs, and the collagen immediately  
254 below the bulbs is less dense.

255 Structure also differed. In wrinkled sheep (Figure 8a) large pieces of very dense

256 collagen (judging by intensity of staining) occur in the lower dermis, and amongst  
257 the follicles. These are presumably bundles of collagen fibrils. In wrinkle-free sheep  
258 (Figure 8b) the collagen has a more layered appearance, and is almost completely  
259 absent from around follicle bulbs. These observations are consistent with the PSR  
260 stained images of Figure 5. The bundles of collagen which show as large continuous  
261 areas in these sections are aligned with the direction of sectioning. Fibre bundles that  
262 have been sectioned across appear as smaller entities. There are fewer large entities in  
263 the wrinkle-free specimens in both Figures 5 and 8. This difference is also discernible  
264 in Figure 2

265 *3.2.3. Type of collagen*

266 One can distinguish Type I and II collagen from size of the bundles of fibrils. For  
267 example in the PSR stained images of Figure 5 the wrinkled specimen clearly has  
268 large bundles of fibrils and therefore a considerable amount of Type I collagen. The  
269 wrinkle-free specimen, however has fewer bundled fibrils, and therefore a lesser amount  
270 of Type I collagen, as seen in Figures 8 and 2.

271 A technique referred to in Section 2, which uses polarised light microscopy was  
272 used to differentiate Type I from Type III collagen. Figure 9 shows two polarised light  
273 images under a 4x objective comparing a wrinkled sheep with a wrinkle-free sheep.

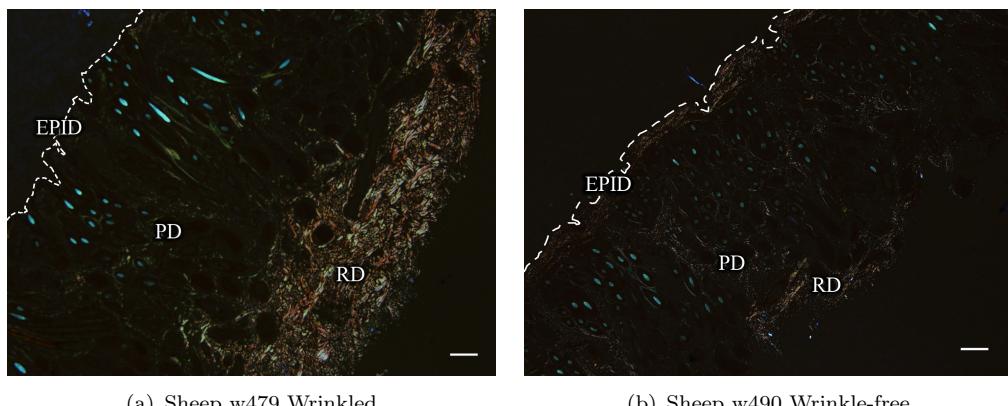
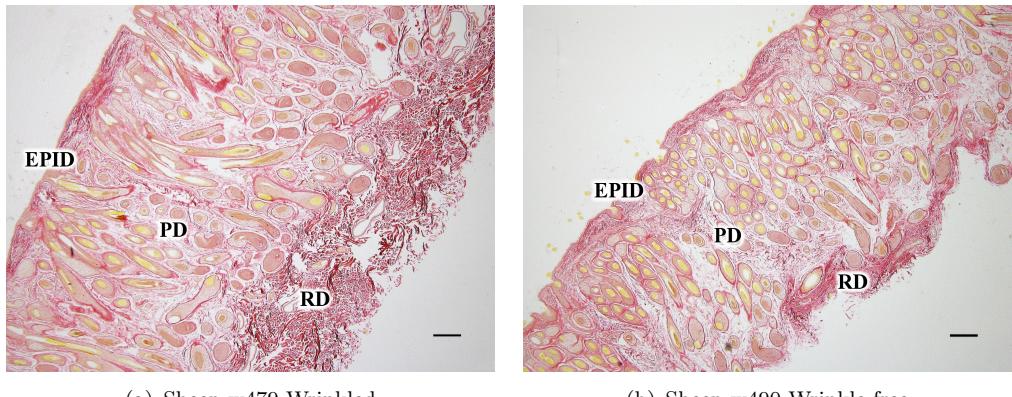


Figure 9.: Vertical sections from a wrinkled (a) and a wrinkle-free (b) sheep from Trial 1 flock 2 stained with PSR and examined with polarised light and a 4x objective. Skin layers are: **EPIID** epidermis, **PD** papillary dermis, and **RD** reticular dermis. Epidermal surface is marked with a dotted line. Scale bar is 200 $\mu$ m.

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

275 Both wrinkled and wrinkle-free specimens have some lower dermal collagen (stained  
276 red with PSR stain in Figure 10), but only the wrinkled specimen shows orange/red  
277 birefringence under polarised light (Figure 9). Because these specimens are from Trial  
278 1, it is possible that some of the lower dermis was removed in trimming the biopsy  
279 specimens. This should not affect comparison of collagen types.

280 It is evident that wrinkled sheep do not just have more collagen, but the extra  
281 collagen is Type I (hard). Wrinkle-free sheep apparently only have Type III (reticular)  
282 collagen. This confirms the conclusion of the previous section from looking at size of  
283 collagen fibre bundles.



(a) Sheep w479 Wrinkled

(b) Sheep w490 Wrinkle-free

Figure 10.: Vertical sections from a wrinkled (a) and a wrinkle-free (b) 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. Skin layers are: **EPID** epidermis, **PD** papillary dermis, and **RD** reticular dermis. Scale bar is  $200\mu m$ .

### 284 3.3. Wrinkle patterns over body

285 All Merinos have small *pin* wrinkles. Pin wrinkles do not seem to form a pattern and  
 286 are uniform across the body. Here, patterns in the large wrinkles which develop from  
 287 birth up to maturity, are discussed. Large wrinkles form a consistent pattern which  
 288 was documented by (Carter 1943). Carter named each wrinkle and associated them  
 289 with successive vertebrae along the spine. Size of wrinkles varies, but not the pattern.  
 290 The pattern is consistent between sheep. Figure 11 shows a photograph of two Merino  
 291 ewes, with and without wrinkle.



Figure 11.: Two Merino ewes from Flock 1 of Trial 2, one wrinkled (left) and one wrinkle-free (right)

292 The wrinkled sheep in Figure 11 is a good example of the pattern to which we

refer. Each wrinkle runs dorso-ventrally, the numbers of wrinkles approximating those of the vertebrae. Each wrinkle appears to mark the position one *dermatome* area of skin (Kirk 1968), with the main nerve from the spine running either under or between wrinkles. We do not know the spatial relationship between wrinkles and nerve channels but it appears to be a one-to-one relation.

Wrinkles on the side of a sheep run vertically. Rows of follicle groups on the side of a sheep run vertically. In mosaic sheep (Fraser and Short 1960), which are somatic fleece mutations, the patterns of mutant fleece run vertically. These phenomena reflect the way skin develops, as a series of separate patches called dermatomes, each patch being associated with one nerve descending from the spine. The reason wrinkle development follows this pattern remains unexplained.

#### 4. Discussion

This study has established from observations on adult Merino sheep that wrinkled sheep have the following:

- more collagen in the lower dermis
- more Type I collagen in the lower dermis
- collagen in the lower dermis extending upwards around follicle bulbs into the upper dermis

Comparison of skin from paired sites on the same sheep, *on-wrinkle* and *off-wrinkle*, has shown that there is no difference in collagen Type or amount. In addition published work has established the following:

- wrinkles have been reported forming in foetal skin of Karakul and Merino lambs at around 100 days of gestation (Bogolyubsky 1940)
- pin wrinkles are small and are present at birth and remain into adulthood. Pin wrinkles are mainly a characteristic of Merino sheep (Carter 1943)
- wrinkles are visible at birth and grow in size as a sheep matures. They are also mainly a characteristic of some Merino sheep. Wrinkles form in a pattern which suggests a one to one relation between wrinkles and dermatomes (Carter 1943)
- large wrinkles consist of epidermis, papillary dermis, and lower or reticular dermis, but not the muscle and fat layers Mitchell et al. (1984)
- collagen is present in the foetal dermis from about day 80, ie at about the same time as when secondary derived follicles are forming (Knight et al. 1993)
- collagen in the dermis gradually becomes more Type I as a sheep matures (Knight et al. 1993)
- 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. (Kozslowski 1966)

Perhaps the most important result above is the negative one. There were no significant histological differences between skin sampled on-wrinkle or off-wrinkle on wrinkled sheep. A wrinkle is therefore not an additional organelle growing on top of the skin; tissues within a wrinkle are exactly the same tissues as in skin in-between wrinkles. A different explanation is required.

We propose two hypotheses which together explain the above observations

##### 4.1. Two layer folding hypothesis

We propose that a wrinkle forms because some layers of skin grow faster than other layers. Any dual layer structure will curve or buckle if one layer changes length or area faster than the other layer, provided the two layers are firmly bound together. A bi-

339 metal strip is one example. In biology, curved surfaces are formed by non-allometric  
340 growth. (Thompson 1917). In ruga mechanics (Diab et al. 2013), dual layered materials  
341 buckle when a stress is applied that causes unequal strains in the layers.

342 We can identify the layers involved. It is known from (Mitchell et al. 1984) that  
343 a wrinkle contains epidermis, papillary dermis and reticular or lower dermis, but not  
344 the muscle and fat layers. The two layers that differ in growth rate are (a) layers 1,2  
345 together, and (b) layers 4 and 5 together. Layer 3 forms a flexible bond between (a)  
346 and (b) . As a sheep matures and wrinkles form, (a) grows faster. Presence of hard  
347 collagen in the lower dermis binds the upper dermis to the muscle and fat layers below.  
348 Hence collagen binds the boundary between (a) and (b), in the same way as the rivets  
349 in a bi-metal strip bind the two layers of metal. If the rivets are loose, the strip does  
350 not curve, if they are tight, it curves.

351 For wrinkles to form there has to also be excessive growth of layer (a) as the sheep  
352 matures. This excess growth of (a) occurs as a result of maturation of the large number  
353 of secondary derived follicles in Merinos. In some Merino sheep without wrinkles excess  
354 growth of layer (a) still occurs, but layer (b) is not bound by hard collagen at the  
355 boundary with layer(a), allowing both layers to expand at different rates. The skin  
356 on such a sheep feels loose and supple. Other breeds of sheep ( eg British breeds) do  
357 not have excessive growth of layer (a) as they mature, so they do not form wrinkles,  
358 regardless of whether they have hard collagen.

359 We know that tiny pin-wrinkles start to form em in-utero at around days 80 to 100.  
360 That is exactly the time window in which the large population of secondary original  
361 follicles is forming in Merino sheep. We suggest that formation of large numbers of  
362 secondary follicles dramatically increases expansion of the epidermis and papillary  
363 dermis, while the lower dermis is held at a slower growth .

#### 364 **4.2. Two factor wrinkle formation hypothesis**

365 Given the above, we suggest that there are two independent factors involved in wrinkle  
366 formation

367 **presence of hard collagen in lower dermis** prevents epidermis and papillary  
368 dermis from expanding independently of the sub-dermis  
369 **excessive growth of papillary dermis** which is probably attributable to develop-  
370 ment of large numbers of secondary follicles and their accessory organs

#### 371 **4.3. Auxiliary issues**

372 The pattern of wrinkles over a sheep's body noted in section 3.3 is not fully understood.  
373 The observation that wrinkles always run in the same direction implies that either  
374 expansion in layer (a) is directional, or collagen binding in layer (b) is directional, or  
375 some other factor interferes to provide a direction. We are not sure, but we favour  
376 the last possibility, because another factor can be identified. We have noted that each  
377 wrinkle occupies one dermatome. A dermatome is an area of skin associated with one  
378 major nerve channel which runs from the spine downwards. The position of the nerve  
379 channel may be involved in deciding where skin is to fold. The major nerve channels  
380 are in the hypodermis, and minor nerves run from there into the dermis, like risers in a  
381 plumbing system. So at the position where the 'risers' cross from hypodermis to dermis  
382 the two layers cannot move independently. At these points the two layers should be  
383 'anchored' together. Rows of such 'anchor points' run from the spine downward. The

384 skin folds parallel to these rows. It is not known whether rows of anchor points are  
385 under wrinkles, or between wrinkles.

386 The hypodermis also contains major blood vessels, both arteries and veins. These  
387 also have minor branches which cross the boundary into the dermis, like risers. Some  
388 information on nerves and blood vessels in sheep skin is given by (Lyne and Hollis  
389 1968), but we have been unable to find the exact arrangement of blood vessels. The  
390 same considerations apply as for nerves, blood vessels may determine 'anchor points'  
391 at which the dermis cannot move against the hypodermis.

392 Development of follicles and development of collagen have a biological connection.  
393 The papilla cells in follicles are differentiated fibroblasts. The fibrocyte cells which  
394 produce collagen fibres are also differentiated fibroblasts. There is an established theory  
395 about the way pre-papilla cells distribute to follicle papillae, and the effects this has  
396 on follicle density and fibre diameters (Moore et al. 1989, 1996). We are unaware of  
397 any similar theory, for collagen. It is possible that the population of fibroblast cells  
398 is limited in number at some stages so that a *tradeoff* situation might exist between  
399 follicle development and collagen development.

#### 400 **4.4. Prediction and verification of hypotheses**

401 To check if the above hypotheses are robust we use them to make one prediction which  
402 we check it against new data.

403 The two factor wrinkle formation hypothesis asserts that for skin wrinkles to form  
404 there must be both hard collagen binding the upper dermis to the hypodermis, and  
405 excessive growth of the upper dermis probably attributable to large numbers of sec-  
406 ondary derived follicles. Under this model, only sheep with both factors present at a  
407 sufficient level will form wrinkles. This implies that the two factors interact. Therefore  
408 we predict that the quantitative genetics of wrinkle will involve an epistatic interac-  
409 tion between the genes for hard collagen and the genes for large number of secondary  
410 follicles. This is something that can be checked.

411 Data from five CSIRO experimental flocks in which degree of wrinkle had been ob-  
412 served according to the photographic standards of Turner, et al. (1953) were available.  
413 These flocks were fully pedigreed and contained a total of 22200 sheep with data.  
414 A mixed model was fitted which removed fixed effects and estimated components  
415 of variance of wrinkle score for individual environment, individual additive genetic,  
416 individual additive x additive epistatic , maternal additive genetic, and maternal envi-  
417 ronmental components, for each flock. Here we just present a summary as a pie chart  
418 in Figure 12 showing average component estimates over the five flocks, as percentages  
419 of total variance.

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

#### 425 **4.5. Further work needed**

426 Points which we were not able to fully investigate.

- 427 – the sheep studied are a small sample of Australian Merinos. A wider study encompassing  
428 diverse strains of sheep and a variety of grazing environments is needed

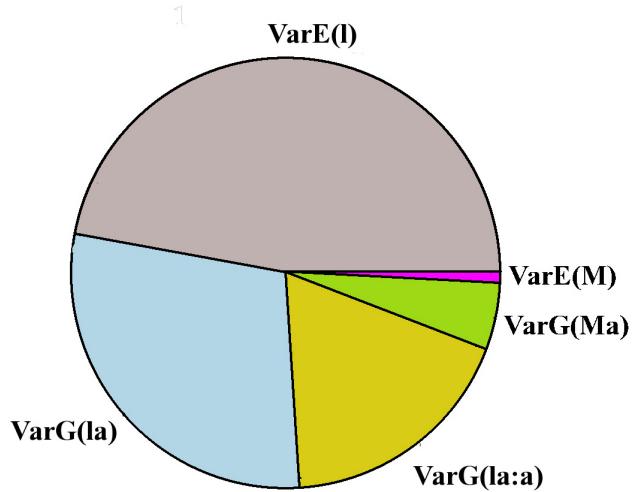


Figure 12.: Summary of analyses of quantitative genetic variation in wrinkle score. The piechart shows percentages of variation attributed to the following variance components: VarE(I) = individual environmental variance, VarG(Ia) = individual additive genetic variance, VarG(Ia:a) = individual additive 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.

- 429 – we studied selected extreme individuals. Do a series of wrinkle grades show the same  
430 relationship with collagen?
- 431 – more sophisticated techniques, such as protein immunochemistry, could help quantify  
432 differences in collagen type
- 433 – association of wrinkle pattern over the body with dermatome pattern needs to be inves-  
434 tigated and its basis determined.
- 435 – do fibroblast cells play a role in determining observed differences in collagen quantity  
436 and type between wrinkled and wrinkle-free sheep?
- 437 – alternatives to our wrinkle formation hypothesis need to be considered.

#### 438 *4.6. Breeding implications*

439 Wrinkle formation in Australian Merino sheep skin is a phenomenon with serious  
440 economic and political consequences. Wrinkled skins ( referred to as *ribbed* in the  
441 leather industry) are not suitable for fellmongering to preserve the skin (Scobie et al.  
442 2005). Wrinkled sheep are more difficult to shear. It has long been known (Seddon et al.  
443 1931) that wrinkled sheep are more susceptible to blowfly strike. Use of the *mulesing*  
444 operation to control flystrike in Merino sheep has recently been subject to intense  
445 animal ethics scrutiny. No practical alternate management option has appeared. The  
446 most effective long term solution would seem to be to breed wrinkle out of Merino  
447 sheep. This approach has at times met with resistance from some Australian Merino  
448 breeders who feel that the extra skin surface area of wrinkled sheep is necessary to  
449 achieve high levels of wool production. This study shows that it is possible to have

450 extra skin surface area without having wrinkle, provided the presence of hard collagen  
451 is avoided.

452 Breeding plans that include some culling on wrinkle usually do not lead to its  
453 complete elimination (for example (Turner et al. 1968)). Quantitative genetic studies  
454 (Hatcher et al. 2012) indicate that it is possible to breed for high wool production and  
455 reduced wrinkle, but these studies ignore the presence of epistatic genetic variance.

456 If the two factor wrinkle formation hypothesis is correct, and if wrinkle really does  
457 exhibit epistatic variation, then breeding to reduce wrinkle by selection on observed  
458 wrinkle scores will have a problem. Such selection would tend to choose both sheep  
459 with few secondary follicles ( low dermal expansion) and sheep with Type III collagen.  
460 Only the latter is desirable, as sheep with few follicles will be poor producers. A careful  
461 implementation of fleece and skin measurements should be able to avoid this issue.

## 462 **5. Conclusion**

463 A wrinkle or skin fold in sheep is not a separate organ or tissue. The tissues within a  
464 wrinkle are the same as the tissues in flat skin. A wrinkle is simply a buckling of skin  
465 caused by differential growth of skin layers.

466 In Merino sheep, skin wrinkles form as a result of an interaction between two skin  
467 layers (dermis and sub-dermis) growing at different rates, and bound together to var-  
468 ious degrees by different grades of collagen. We suggest that the upper dermis grows  
469 faster than other skin layers in wrinkled Merino sheep, because of the development of  
470 large numbers of secondary follicles.

471 Type and amount of collagen in the lower dermis have a strong association with  
472 wrinkle formation.

473 One might breed a wrinkle-free Merino by reducing the number of secondary follicles,  
474 but that would adversely affect wool production. An alternative seems to be to breed  
475 wrinkle-free Merinos by changing the type of collagen, so that the expanding upper  
476 dermis is not strongly bound to the slower growing sub-dermal tissue layers.

## 477 **Acknowledgement(s)**

478 The authors are indebted to Dr N. Donovan, Dr. G. P. M. Moore and Dr. P. G.  
479 Swan for advice and revision of the manuscript. We thank Mrs. S. Watts and Mr.  
480 S. Gordon for material support. We thank the Histopathology Department of the  
481 Faculty of Medicine and Health, University of Sydney for collaborative assistance with  
482 histological observations.

## 483 **Disclosure statement**

484 Dr Jim Watts was founder of the SRS breeding system for Merino sheep. Mr Jim  
485 Gordon is a breeder and classer of Merino sheep, but is not associated with SRS  
486 Genetics. The other authors have no association with SRS.

487 **Data availability statement**

488 The data that support the findings of this study are openly available in figshare at  
489 <http://doi.org/10.6084/m9.figshare.12318473>

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