Vol. X (1—4) 1973.

A QUANTITATIVE METHOD FOR THE STUDY OF BUFFALO SKIN STRUCTURES

M.A. MAJEED[1], M.D. AHMAD[2], M.B. SIAL[2] & M. AJMAL[1]

A comprehensive method for the quantitative study of 19 different parameters of skin is described. It consists of sampling, processing, staining, enumeration and other evaluation procedures. Three of these parameters pertain to thickness measurements, three to frequency estimates, four each to ordinary and giant type of hair follicles, and the remaining five envisage sweat gland measurements.

A trephine is designed which can be used on a living buffalo or on her detached skin. It can punch circular samples of approximately 1 cm^2 surface area each. Samples of this size are not only easy to manipulate but also help in enlarging the scope of the study and enhancing the reliability specially of its frequency estimates. A method for the estimation of surface area of a whole detached skin is also devised.

INTRODUCTION

Skin structures of the European (*Bos taurus*) and tropical (*Bos indicus*) cattle have been shown to differ so much in their per unit area frequency, form and even location that many of these features are now considered important to their overall heat balance (Bianca, 1965). Some of these characteristics have, also been used in tracing the origin of different breeds of cattle, their inter-relationships and their overall classification (Jenkinson and Nay, 1968, 1972 and 1973) Their contemplated use in the selection of dairy cattle (Nay and Jenkinson, 1954) has prompted their detailed study in other dairy animals, e.g., the domestic buffalo (*Bos bubalis*).

Quantitative findings can be misleading until and unless all the parameters studied are clearly defined and standardized sampling, processing, enumeration and calibration procedures are adopted, An attempt is, therefore, made to define the more commonly studied parameters of skin and to describe their methods of study in this fast emerging dairy animal of the East.

DEFINITIONS AND METHODOLOGY

Different procedures are described in the same order in which it is convenient to proceed. The same sequence may, therefore, be maintained.

1. Faculty of Veterinary Science 2. Faculty of Animal Husbandry, University of Agriculture, Lyallpur.

A. Surface Area

Each whole skin is weighed soon after the slaughter of the animal (W_1). Rectangular pieces of exactly 15×5 cm size are obtained from all the different locations to be studied and their composite weight (W_2) taken. Surface area of each whole skin is then estimated by the formula :

$$A_1 = \frac{A_2 \times W_1}{W_2}$$

where :

A_1 = Estimated surface area of a whole skin.

A_2 = Area of n number of rectangular pieces of skin together, or n (15 × 5) cm^2.

W_1 = Total weight of a whole skin.

W_2 = Composite weight of n number of rectangular skin pieces together.

B. Thickness Measurements

Gross skin thickness (GST) is measured by a vernier calliper and is expressed in millimeteres (mm). All superfluous hair, subcutaneous fat and cutaneous muscle removed rectangular pieces of skin are double folded and thickness readings taken at five random points. Mean of these measurements is considered typical GST measurement for the location.

Total epidermal thickness (TET) is the mean shortest distance between the bottom of any five first encountered downgrowths or pegs and the free surface of skin. Following Hafez *et al.* (1955), TET is subdivided into main and papillomatous portions. The ratio between the two, expressed as percentage : $\frac{\text{main part} \times 100}{\text{papillomatous part}}$ is termed per cent main epidermis (PCME).

Both the parts of TET are measured with the help of a previously calibrated eye-piece micrometer scale (Nay and Hayman, 1956) and the readings converted to microns (μ). Stained 25 u thick serial vertical sections of skin are used only at points where the stratum corneum is intact. Oblique sections of skin in which the hair follicles do not show typical bulbs at their roots, are all ignored.

C. Sampling and Fixation Procedures

Each denuded rectangular skin piece of 15×5 cm size is appended hair side down, on a 1 cm thick deodar-wood slab of the same size and left

in neutral formalin. Four drawing pins, one occupying each corner, are used to secure the piece. Each of these skin pieces is disengaged the following day, held securely over a larger yet thicker pad of firm synthetic foam and cut into a number of circular skin pieces by successive vertical thrusts of an electric drill driven trephine (Plate I). Overnight stay of the relatively thicker skin samples in neutral formalin cause considerable expansion of their loose dermis. As the greater part of the corium below the follicle papillae is not required for study, it is trimmed away after Carter and Dowling (1954). A sharp pair of large scissors is used to carefully bisect the desired number of circular skin pieces in the exact direction of the hair stubbles. All uneven and out of shape circular and semi-circular skin pieces are discarded and the rest placed in fresh neutral formalin for another 24 hours.

D. Processing and Staining Techniques

All the thus fixed skin pieces are washed in running water for over two hours. Dehydration is done for two hours in each of the usual grades of ethanol. Clearing is done in two changes of xylene. A period of two hours, in either of the two changes of melted paraffin (M.P. 56°C) in a vacuum oven under 20 lbs of pressure, is absolutely necessary for proper impregnation. Embedding is done in such a way that the straight ends of the semicircular skin pieces and the epidermal side of the circular ones could finally face the microtome knife.

Both 25μ or 8 μ thick, vertical and horizontal serial sections are obtained respectively from the semicircular and the circular skin pieces. A rotary microtome provided with an indicator dial (A O Spencer Model 820) is used for cutting the sections. To facilitate even spreading, appropriate sized ribbons are made to float on warm water at 50°C for about 10 minutes in a thermostatically controlled water bath. All these sections are picked up on Mayer's albumen applied glass slides which are made to dry on a thermostatically controlled hot plate for two hours.

The choice of the staining procedures rests on the parameters to be studied. If the rate of cutaneous pigmentation is to be determined the decerated 25 μ thick serial vertical sections are mounted in canada balsam without staining (Goldsberry and Calhoun, 1951). Aqueous Celestine blue (Humason, 1966) treated sections when stained with Van Gieson stain (Jones, 1966) aid in distinguishing between the various skin structures which promote their precise measurements. The routine hematoxyline and eosin stain (Humason, 1966) is yet another easily available alternative.

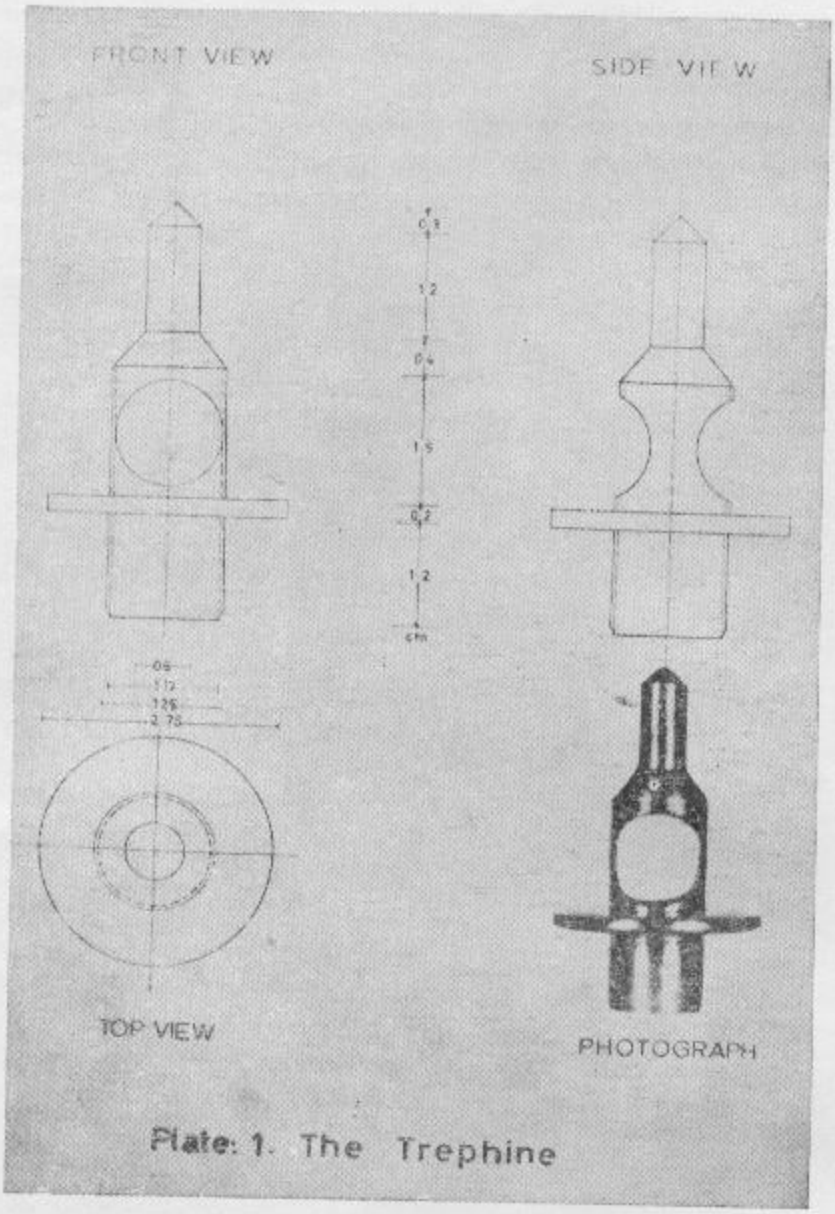

Plate: 1. The Trephine

E. Frequency Estimates

The sparse hair of the buffalo are known to be of two distinct sizes (Yamane and Ono, 1936; Hafez *et al.*, 1955). Hair follicles showing a diameter of 200 μ or more are arbitrarily classified as giant follicles (GF) as against all others which are termed the ordinary follicles (OF). Because the

area of each circular skin piece is approximately 1 cm^2 the number of hair follicles encountered in each of its intact horizontal sections is directly taken as total hair follicle frequency (THFF) per cm^2 of the unprocessed skin. Following Yamane and Ono (1936) and Hafez *et al.* (1955). THFF is also considered to be an estimate of total sweat gland frequency (TSGF). The ratio between giant follicle frequency (GFF) and THFF when expressed in percentage $\frac{GFF \times 100}{THFF}$ is termed per cent giant follicles (PCGF).

The 6x eye piece of an ordinary light microscope is furnished with a squared 1 cm^2 graticule. Its superimposed image on the slide not only facilitates the distinction between the two types of the hair follicles but also guides the eye, at the time of the count, in the same manner as the squares of a hemocytometer guide the count of the blood cells. Stained 25 μ thick serial horizontal sections are scanned under the low power : once for recording the THFF and a second time for taking the GFF.

F. Hair Follicle Measurements

Length (L), depth (D) and diameter (DM) of the hair follicles are separately measured in any 20 and any 10 first encountered hair follicles of ordinary (OF) and giant (GF) types, respectively. All these measurements are taken in microns (μ) with the help of an eye piece micrometer scale after Nay and Hayman (1956) but in accordance with the definitions of Jenkinson and Nay (1968). Their respective angle of slope (AS) are, however, determined by trigonometry (Jenkinson and Nay, 1972). Only the full length visible longitudinal sections of the hair follicles are gauged for taking L, D and AS measurements. These measurements are taken from stained serial 25 μ thick, vertical sections of skin. Readings on DM are taken from stained serial horizontal sections of the same thickness. No oblique section of the hair follicle, vertical or horizontal, is calibrated. Means of all these measurements are recorded as typical values of the parameters in question.

G. Sweat Gland Measurements

Length (L), diameter (DM) and volume (V) of the sweat glands (SG) are defined after Nay and Jenkinson (1964) but their depth (D) is taken in accordance with the definition of Nay and Hayman (1956). One reading is taken on each of the 10 first observable sweat glands for SGL and SGD, while three readings per gland are recorded in case of SGDM (Nay and Hayman, 1956). Ten random glands are thus calibrated and the mean taken as the typical value of the parameter in question. Only the maximally exposed

longitudinal sections of the unmutilated sweat glands are calibrated from 25 μ thick, stained, serial, vertical sections. Distorted, collapsed or incompletely visible glands are ignored.

Volume (V) of the sweat glands is determined by the formula :

$$SGV = \pi \times \frac{1}{4} SGDM^2 \times SGL$$

and is expressed in $\mu^3 \times 10^6$ (Nay and Jenkinson, 1964).

RESULTS AND DISCUSSION

The technique described in the preceeding sections was developed and tested during the course of a recently concluded study in which attempt was made to ascertain some quantitative aspects of the indigenous buffalo heifer skin (Majeed *et al.*, 1973). It is on the basis of this experience that the said procedures are being discussed.

Several physiological factors are known to influence the overall size of skin in animals. As such its calibration in the living state is usually not easy. The proposed method estimates it from its total weight which is not difficult to take in the detached skins. Moreover, it aims at excluding some of the transitory variations in skin size, e.g., those due to excitement, climatic factors and the like. This method, however, cannot be used in the living animals.

Proper calibration of any structure would require not only a clear view of the whole structure in its normal form and natural state but would also need reliable means to do so. Many of the skin structures studied being sub-microscopic cannot be calibrated untill and unless the tissue has been cut into thin sections and the structure in question differentially stained. But both these processes can disturb the original form and natural size of the soft skin structures.

Well known curling of the skin tissue during processing may affect the ultimate size of different samples so variably that a fresh correction factor may have to be worked out, for each and every sample, if the frequency estimates made on a fraction of such a section are to be projected over the whole body. Blotters worked well when stappled around the fine skins of fur animals, as well as, sheep (Schied, 1954) but gave in when applied around comparatively thicker skin of the buffalo. Sufficient flattening cum hardening effect is, however, produced by affixing the rectangular pieces of buffalo heifer skin on wooden slabs. This facilitates smooth punching which gives added accuracy particularly to the frequency estimates. Denuded state and the hair side down position of the skin at the time of the first fixation hastens the desired effect.

Hand operated biopsy punch of 1 cm internal diameter (Carter and Dowling, 1954), and electric-drill-driven hollow ended steel bits of 0.2 and 0.4 cm (Evans et al., 1957), or 0.373 cm (Findlay and Jenkinson, 1960) internal diameter have all been used for obtaining the biospecimens of skin in cattle. But only a fraction of the thus obtained samples is calibrated and a correction factor is applied which has been said to satisfactorily account for the shrinkage in the course of processing. However, for the thinly haired buffalo skin (Hafez *et al.* 1955 and Majeed *et al.*, 1973) this size of the sample is neither easy to orientate at the time of bisecting the circular skin piece nor the scanning of its small portions can give satisfactory estimates of the total hair follicle frequency (THFF).

In order to obtain samples of manageable and reasonable size a trephine with an internal diameter of 1.13 cm is designed (Plate 1). It is worked by an electric drill capable of making 3000 to 5000 rpm. Too deep penetrations are guarded against by its built-in shoulders and the upper hole on its body facilitates its evacuation. As each intact horizontal section of the circular skin piece is scanned in full and the readings directly taken as estimates of THFF or TSGF per cm^2 of the unprocessed skin, consideration of shrinkage becomes unnecessary. Furthermore the much larger sample size not only improves the reliability but also facilitates manoeuvreability particularly at the time of bisecting and trimming.

All skin processing methods advocate special care in following the direction of the hair at the time of preparing vertical sections. This is because in this way one is likely to get the maximum number of full length visible longitudinal sections of the hair accompanying sweat glands. Obviously, their oblique sections are likely to give low readings of their true measurements. A good sectioning procedure must, therefore, ensure that majority of its cuts are parallel to the direction of the hair. This is all the more important for the thinly haired skin of the buffalo (Hafez *et al.*, 1955; Majeed *et al.*, 1973) in which case each sweat gland lost in processing affects the limited size of the sample more adversely.

Sections cut by hand after Nay and Hayman (1956) from the unembedded skin pieces are in most cases neither of constant thickness nor of uniform quality. An uneven pressure on the razor blade may disturb the crucial direction of the cuts or disfigure the structures under study. An unequal drying may likewise disturb the gland size differently. Several of the processing, staining and mounting steps in all the invogue techniques bring

different grades of ethyl alcohol in direct contact with the skin tissue. The resultant dehydration may reduce the overall size of the soft skin structures or modify their shapes. All the same, so far there seems to be no escape either from these procedures or from these reagents.

Some of the more salient sources of variation in different steps of sampling, processing, staining and calibration methods are thus brought out. This should not in any way suggest that the technique under discussion can exclude all this variability. All what it aims at is minimizing this variation or making it uniform by (i) enlarging the sample size, (ii) increasing the number of observations, and (iii) by consistently following a preset routine. Following steps are particularly helpful in achieving this objective:

(a) Application of rectangular skin pieces on wooden slabs and constant use of the same fixative for identical length of time reduces the shrinkage and makes it less variable.

(b) Large sized circular skin pieces facilitate trimming and bisecting steps: making the all crucial first cut parallel to the direction of the hair stubbles.

(c) Embedding in paraffin and cutting by a microtome help in maintaining this direction in all the subsequent cuts and in saving the natural shape of the sweat glands.

(d) Identical processsing, staining and calibration procedure, specially the reagents used, timings observed and the conditions worked in add to the much needed uniformity.

(e) A fixed schedule of spreading the ribbons and drying the sections at constant temperatures for identical periods of time helps reduce this source of variation.

(f) Consistent use of the same differential stain on the serial sections of the same thickness enhance the precision.

(g) Rejection of all the out of shape samples at various stages of the procedure makes the experimental materials more homogeneous.

LITERATURE CITED

Bianca, W. 1965. Reviews of the progress of dairy science. Section A. Physiology. Cattle in hot environment. J. Dairy Res., 32: 291—345.

Carter, H.B. and D.F. Dowling. 1954. The hair follicle and apocrine gland population of cattle skin. Aust. J. Agri. Res., 5(4) : 745—754.

Evans, C.L., A.M. Nisbet and K.A. Ross. 1957. A histological study of the sweat glands of normal and dry coated horses. J. Comp. Path. & Therap., 67 (4) : 397—405.

Findlay, J.D. and D. McEwan Jenkinson. 1960. The morphology of bovine sweat glands and the effect of heat on the sweat glands of the Ayrshire calf. J. Agri. Sci., Camb. 55(2) : 247—249.

Goldsberry, S. and M.L. Calhoun. 1951. The comparative histology of the skin of Herford and Aberdeen Angus cattle, Amer. J. Vet. Res., 207(4) : 61—68.

Hafez, E.S.E., A.L. Badreldin and M.M. Shafei. 1955. Skin structure of Egyptian buffaloes and cattle with particular reference to sweat glands. J. Agri. Sci., Camb., 46(1) : 19—30.

Humason, G.L. 1966. Animal Tissue Techniques. 2nd ed., W.H. Freeman & Co., San Francisco.

Jenkinson, D. McEwan and T. Nay. 1968. Sweat gland and hair follicle measurements as indicators of skin type in cattle. Aust. J. Biol. Sci., 21: 1001—1011.

Jenkinson, D. McEwan and T. Nay. 1972. The sweat glands and hair follicles of European cattle. Aust. J. Biol. Sci., 26 : 251–275.

Jenkinson, D. McEwan and T. Nay. 1973. The sweat glands and hair follicles of Asian, African and South American Cattle. Aust. J. Biol. Sci., 26: 259—275.

Jones, R. McClung. 1966. Basic Microscopic Technics. The University of Chicago Press, Chicago.

Majeed, M.A., M.B. Sial, M. Ajmal and M.D. Ahmad. 1973. Quantitative data of Nili-Ravi buffalo heifers in summer. IRCS (73-8) 45-1-4.

Nay, T. and R.H. Hayman. 1956. Sweat glands in Zebu (*Bos indicus L*) and European (*Bos taurus L*) cattle. I. Size of individual glands, the denseness of their depth below the skin surface. Aust. J. Agri. Res., 7(5) : 482—494.

Nay, T, and D. McEwan Jenkinson. 1964. Skin structure and milk production of British dairy cattle. J. Dairy Res., 31(1) : 53–58.

Schied, R.J. 1964. Minimizing curling of skin biopsies by blotting paper-supports during fixation. Stain Technology, 39(4) : 196.

Yamane, J. and Ono, Y. 1936. Rassenanatomische Untersuchungen der Hautstruktur vom Buffel, Zebu, Formosarind und Friesisch-Hollander im Hinblick auf das Problem der Tropenanpassung. Mem. Fac. Sci. Agri. Taihoku 19 : 87—136.