DIGITAL ANALYSIS OF EYE PIGMENTATION OF HEREFORD, HEREFORD × *BOS INDICUS* OR HEREFORD × *BOS TAURUS* CATTLE

A Thesis

by

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ABSTRACT

Ocular neoplasia in cattle with white faces contributes to large production losses. It occurs less frequently in cattle with pigmented eyelids. Corneoscleral pigmentation (irregular extension of color from the iris into the sclera) is related to eyelid pigmentation and possibly ocular neoplasia. The objectives of this study were to evaluate eyelid and corneoscleral pigmentation using digital images and objective quantification of pixel intensities (the color of eyelid and corneoscleral regions within the image) to obtain a proportion of eyelid and corneoscleral pigment. Hereford, Hereford × *Bos indicus* and Hereford × *Bos taurus* animals were included in the study ($n = 1083$). Upper and lower eyelid pigment proportions and corneoscleral pigment proportions were determined independently. Fixed effects included breed type, age categories and sex of the animal. Lesion presence (1) or absence (0) was obtained by visual appraisal and was assumed binomially distributed. Left eyelid pigmentation proportions (averaged for upper and lower eyelids) for Hereford (0.67 ± 0.021) were significantly lower than *Bos indicus* (0.94 ± 0.011) or *Bos taurus* (0.92 ± 0.011) crosses. Right eyelid pigmentation proportions (averaged for upper and lower eyelids) differed ($P < 0.05$) for Hereford (0.68 ± 0.022), *Bos indicus* (0.94 ± 0.011) and *Bos taurus* (0.90 ± 0.011). Corneoscleral pigmentation proportions (averaged for left and right eyes) differed for each of the three breed types, Hereford (0.05 ± 0.034), *Bos indicus* (0.32 ± 0.018) and *Bos taurus* (0.40 ± 0.016). Mature animals (0.34 ± 0.017) had a significantly greater proportion of corneoscleral pigment than did the calves and yearlings (0.17 ±

0.018). Proportion of lesion presence for Hereford (0.07 ± 0.022) was significantly more than *Bos indicus* (0.01 ± 0.005) or *Bos taurus* (0.01 ± 0.003). By age category, mature animals (0.06 ± 0.011) had significantly greater presence of lesions than did the calves and yearlings (0.01 ± 0.002). There were strong positive correlations for upper and lower eyelid, between left and right eyelids and between eyelid and corneoscleral pigmentation. Crossbreeding with *Bos taurus* or *Bos indicus* animals increase pigmentation and possibly inhibit incidence of ocular neoplasia.

DEDICATION

So do this my son, to free yourself: Go- to the point of exhaustion- Allow no sleep to your eyes, no slumber to your eyelids. Free yourself, like a gazelle from the hand of the hunter, like a bird from the snare of the fowler.

(NIV Prov. 6. 3-5)

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INTRODUCTION

Production losses in Hereford cattle due to bovine ocular squamous cell carcinoma (commonly called “cancer eye”) are a major concern in the beef industry primarily because of condemned carcasses at slaughter and shortened productive life of affected animals (Merial, 2008). Anderson et al. (1957) and Anderson (1960, 1963, 1991) reported lower incidence of bovine ocular squamous cell carcinoma in white faced cattle with increased pigmentation around the eyes as compared to cattle without pigmentation around the eyes. Updated characterization of eye pigmentation, including eyelid pigment and corneoscleral pigment- the pigment on the eyeball in or near the corneoscleral junction, in Hereford straightbreds and crossbreds could facilitate identification of responsible genomic regions by providing for more precise phenotypes. Objectives of this study were to 1) formulate a modern protocol for assessing eyelid and corneoscleral pigmentation in Hereford, Hereford × *Bos taurus* and Hereford × *Bos indicus* cross white faced animals via utilization of digital cameras and computer programs; 2) assess variation in eyelid pigmentation-based on this protocol; and 3) assess variation in corneoscleral pigmentation in these animals; 4) confirm or refute the former indications that pigmentation affects carcinoma frequencies in white faced cattle.

LITERATURE REVIEW

Bovine ocular squamous cell carcinoma

There are specific locations where bovine ocular squamous cell carcinoma is common, namely, the eyelid and the eyeball, as there are various factors related to this carcinoma. Bovine ocular squamous cell carcinoma is a skin cancer occurring on the eyelids, eyeball, nictitating membrane or caruncle of cattle, most common in the Hereford breed, frequently known as “cancer eye” (Anderson et al., 1957). This carcinoma in cattle may be affected by environmental factors, bone structure and other physical attributes, heritability, nutrition, pigmentation around the eye, and age (Anderson, 1991), and some evidence supports the role of a viral precursor (Merial, 2008). The lesions associated with cancer eye are similar to tumors of a viral origin; the papillomavirus may be involved (Newton, 1996), however, there are many other causal factors (Pfister, 2003).

There are 2 distinct areas of the eye where most precancerous to cancerous lesions occur. These 2 regions are the eyelid and eyeball, which includes the corneoscleral junction, the nictitating membrane (third eyelid), and caruncle, (Figure 1). The corneoscleral junction is the area where the cornea and sclera meet. The cornea is the layer above the iris and pupil, and the sclera is the white of the eye beneath the protective conjunctiva.

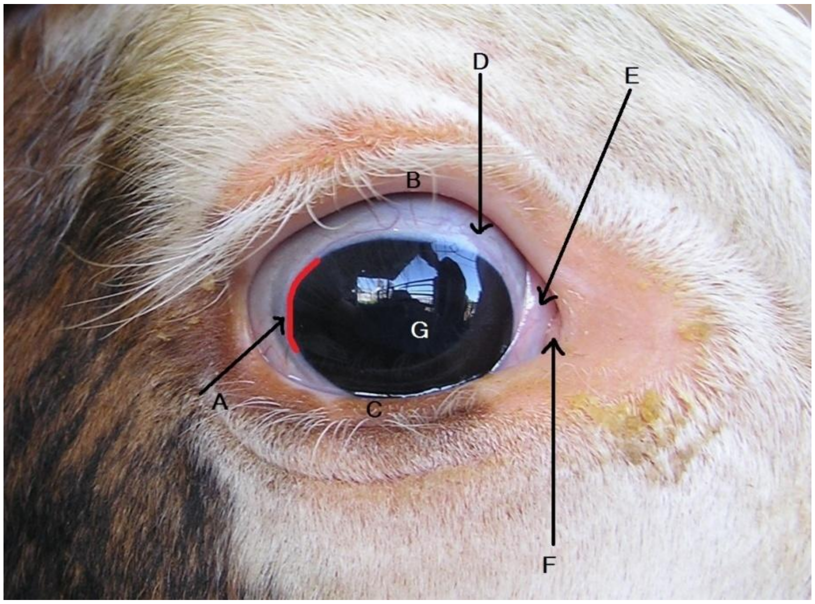

Figure 1. Diagram of bovine eye.

A. The red line indicates the corneoscleral region, which encircles the iris (colored part of eye); B. Upper eyelid; C. Lower eyelid; D. conjunctiva (protective layer above the sclera); E. nictitating membrane (third eyelid); F. lacrimal caruncle; G. cornea (transparent layer above the iris and pupil). (Adapted from McCracken et al., 1999; photo by Kaycee Davis)

Lesions on the eyeball, nictitating membrane, and caruncle can include precursors such as plaque (epithelial hyperplasia), papilloma (small wart-like growth on the skin), or both. The plaque stage is the initial precursor to pathogenesis of squamous carcinoma (Anderson et al., 1957). The papilloma lesion is considered the intermediate state in pathogenesis, typically on the eyeball, nictitating membrane, or caruncle, with the most common occurrence on the corneoscleral junction (Anderson et al., 1957). For lesions on the eyelid, a keratoma (callus or thick epidermal growth) can develop in areas of keratosis and acanthoses (diffuse epidermal hyperplasia). Eyelid lesions can sometimes go through the papilloma stage before becoming cancerous, but the most common pathway begins as a keratoma. The occurrence of a keratoma is considered to be a precursor to squamous carcinoma (Anderson et al., 1957). Keratomas occur most frequently near the hairline in older animals, and are generally dirty brown in appearance. The most common of the four main types of lesions (Figure 2) are lesions of the eyeball, specifically in the corneoscleral junction.

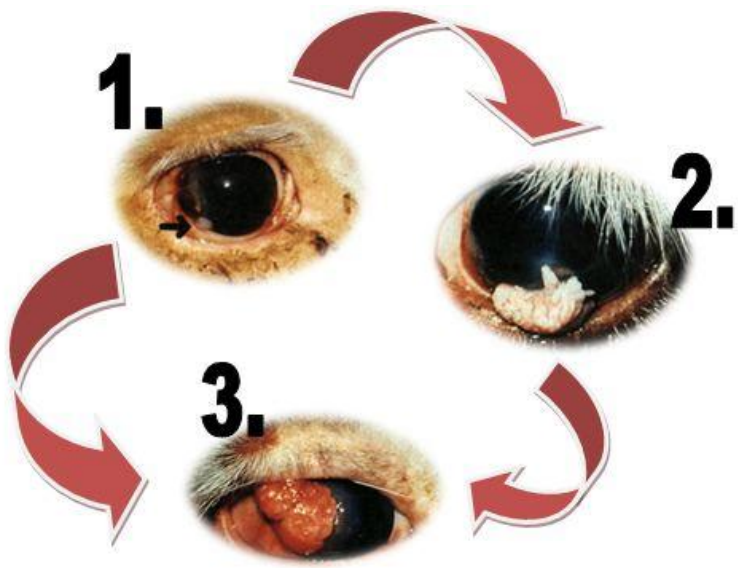

Figure 2. Progression of carcinoma of the eyeball. Plaque build-up on the eyeball; 2. Papilloma formation; 3. Cancerous lesion (Adapted from Gardiner et al. 2004)

Lesion occurrence

Woodward and Knapp (1950) reported in a purebred Hereford herd a significantly higher incidence of bovine ocular squamous cell carcinoma at 5 years of age and again at 7 yr of age, increasing with cow age in general. At ages 5 and 7 the number of cows affected by bovine ocular squamous cell carcinoma or its precursor lesions was remarkably high and increased only slightly at the older ages (Table 1).

Table 1. Incidence of eye cancer and age of disposal of cows[1]

Age of cow in years	Number	Incidence
3	2	0.1
4	3	0.2
5	13	1.4
6	8	1.0
7	14	2.2
8	11	2.2
9	9	2.3
10	9	2.4
11	4	3.5

[1]Adapted from Woodward and Knapp (1950).

Bovine ocular squamous cell carcinoma inheritance

Woodward and Knapp (1950) reported of the 73 cows that developed bovine ocular squamous cell carcinoma in the multi-year study, 14 were mother-and-daughter pairs. This suggests additive genetic control of this trait. Heritability can be explained as the measure of strength of the relationship between performance and breeding value for a trait in a population (Bourdon, 2000). Heritability can also be defined as how good a predictor the genotype is of the phenotype; it is additive genetic variance as a proportion of additive plus error phenotypic variance. The heritability of bovine ocular squamous cell carcinoma was estimated to be 0.4 by Woodward and Knapp (1950). Anderson (1991) concurred and presented estimates of heritability from 0.27 to 0.30 for number of tumors in a review of multiple studies (Blackwell et al., 1956; Anderson, 1959; Russell et al., 1976). The incidence of bovine ocular squamous cell carcinoma and multiple lesions in one or both eyes of the same cow is much less likely than having a single episode of bovine ocular squamous cell carcinoma in one eye. Bovine ocular squamous cell carcinoma may be contracted more than once, especially when the first occurrence of bovine ocular squamous cell carcinoma happens before 7 years of age (Merial, 2008). However, affected animals older than 7 may not be kept in the herd long enough to have a recurrence.

Pigmentation of eyes and cancer

Anderson (1960) demonstrated the relationship between eyelid pigmentation (also known as circumocular pigmentation, the pigmentation of skin around the eye) and decreased incidence of eyelid lesions in Hereford cattle (Table 2). Anderson (1960) observed no lesions in any pigmented area of the lids, regardless of the darkness of red pigment.

Table 2. Observed and expected frequencies of ocular squamous carcinoma and its benign precursor lesions in animals with increased average amounts of circumocular pigmentation[1]

		Percent pigmentation							
Condition of lids		0	1-20	21-40	41-60	61-80	81-100	Total	Chi Square
Lid lesions	Observed	14	17	9	7	3	0	50	
	Expected	8.5	10.0	10.5	7.5	5.0	8.5		
Normal lids (without lesions)	Observed	4	4	13	9	7	18		
	Expected	9.5	11.0	11.5	8.5	5.0	9.5		
Total		18	21	22	16	10	18	105	

[1]Adapted from Anderson (1960).

***P* < 0.001

Inheritance of pigmentation

Most cattle with white faces have Hereford in their pedigree; they are at least heterozygous at the Hereford spotting pattern locus. Figure 3 gives an indication of the possible extreme phenotypes; both are considered to have the white face phenotype.

Figure 3. Examples of white face phenotype. Left minimally marked white face, right almost fully marked white face both animals are at least heterozygous for the Hereford spotting pattern (Photos by Kaycee Davis)

Pitt (1920) presented some of the earliest documented observations of eye pigmentation and color patterns in Hereford cattle and corresponding speculations about their inheritance. Pitt (1920) noted the preference among cattle breeders in Jamaica for cattle with pigment "around the eyes on account of their supposed immunity to the attacks of flies and certain eye diseases." Pitt (1920) presumed that the presence of pigment around the eye was controlled by a single locus, "R," with incomplete dominant gene action, since heterozygotes (Rr) had reduced amount of pigment present, but were not entirely different from the homozygous (RR), pigmented eyelid or as Pitt refers to them, "red-eyed," counterparts. Pitt believed that cattle that exhibited extended quantity of red relative to white (e.g., on the neck, what Pitt called "dark neck" and attributed to a distinct locus, "D"), had greater quantity of red color around the eyes as well. Selection at the time may have been primarily based on color and preservation of the distinct

Hereford pattern, which would have avoided excessive amounts of red and therefore, pigmentation around eyes.

Figure 4 illustrates Pitt's notion of the correspondence of single gene action with variations of the pigmentation around the eyes, as well as the belief in the association of quantity of red (especially on the neck) with degree of circumocular pigmentation.

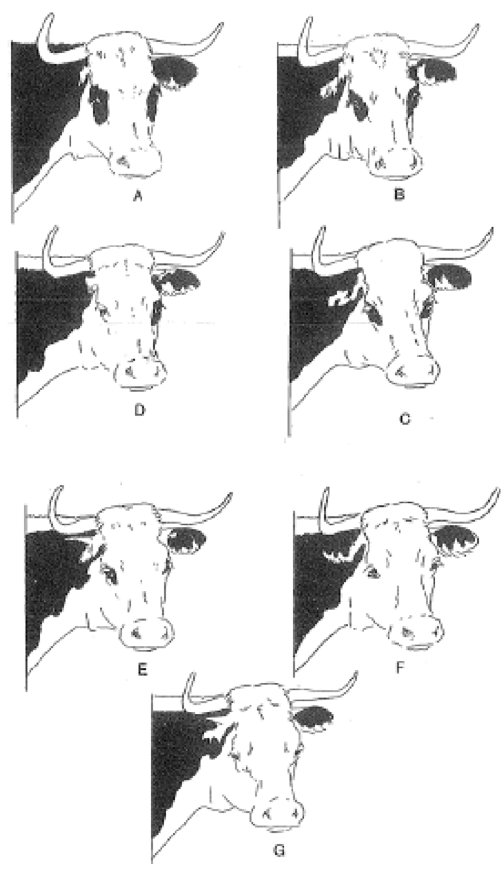

Figure 4. Variation of pigmentation in the Hereford pattern. A being heavily pigmented, through G being least pigmented (reproduced from Pitt 1920)

The most extensive characterization of bovine ocular squamous cell carcinoma and related factors from Anderson et al., (1957) and Anderson (1960) concluded a high degree of genetic control for the proportion of eyelid pigmentation; estimates of heritability from that work ranged from 0.4 (Anderson et al., 1957) to 0.46 (Anderson, 1960).

Corneoscleral pigmentation

Anderson (1991) reviewed long-term investigation in 2,831 Herefords and concluded that eyelid and corneoscleral pigmentation were heritable and genetically correlated (Blackwell et al., 1956; Anderson et al., 1957; Anderson, 1959, 1963; Anderson and Skinner, 1961). Selection for darker eyelid pigmentation often results in darker eye color (Vogt et al., 1963). Corneoscleral pigmentation is the color surrounding the iris and cornea on the eyeball at the corneoscleral junction. Corneoscleral pigment is not sufficiently developed to provide an accurate measurement until sometime after 5 yr of age (Vogt et al., 1963), and may increase in area substantially after that time, and again after 9 yr of age. These ages were detailed as times of increased risk of occurrence of carcinoma (Table 1; Woodward and Knapp, 1950).

Increases in corneoscleral pigmentation have been associated with decreases in lesion frequencies in Hereford cattle (Vogt et al., 1963). Lesions developed in previously unpigmented areas of the eyeball have been shown to be progressively surrounded by corneoscleral pigmentation, possibly as a defense mechanism (Anderson, 1991). Anderson (1991) repeatedly observed that lesions seemed to initiate or evoke the appearance of corneoscleral pigment, resulting in the appearance that a lesion has formed in a pigmented area. Although older cattle may be more susceptible to bovine ocular squamous cell carcinoma, they may also have increasing amounts of corneoscleral pigmentation; it is intriguing that lesion occurrence and corneoscleral pigment may be related.

Bovine genome and the spotting locus

The Hereford pattern is known as a piebald pattern, a spotting pattern of large areas without pigment, with skin colored similarly to the surrounding hair color. Piebaldism is irregular and asymmetrical in form, and some animals may exhibit color of irises based on the presence or absence of surrounding pigment (Olson, 1981). The spotting locus in cattle "S" is on BTA6 (Grosz and MacNeil, 1999). This gene causes white spotting, and may be homologous to one of several coat color loci on mouse MUR5 (Grosz and MacNeil, 1999). Three loci, including tyrosine kinase membrane receptor (Kit), patch (Ph) and rump white (Rw) are all associated with coat color and located on MUR5, which corresponds to BTA6. Mutations of these three genes resembled mutations at the bovine S locus (Grosz and MacNeil, 1999). The Kit gene is known to be involved with 3 populations of migratory stem cells: neural-crest derived melanocytes, hematopoietic stem cells, and primordial germ cells (Fleischman, 1993). Grosz and MacNeil (1999) reported the Kit oncogene (gene that can cause a cell to become malignant) may be responsible for mutations in the spotting locus causing anemia or ocular squamous cell carcinoma in the Hereford breed.

MATERIALS AND METHODS

Data collection

Data, including images and pedigree information, were collected from cows, calves, and bulls at various facilities across the Southern United States (Table 3).

Cattle were caught by head in a working chute. The eye area was cleaned if necessary. One image per eye with both eyelids and the corneoscleral region clearly visible was desired, however using live animals, this was often difficult, so multiple images were taken to ensure at least one adequately composed image of each eyelid. Analyzed photos included two pictures each of the right and left eye areas: one straight across and one aiming up (to ensure the eyelid under the eyelashes was visible). The camera used for most images was an Olympus Camedia C-740 digital (Olympus Company of the Americas, Center Valley, PA) with 3.2 megapixels and 10× optical zoom.

Table 3. Location and count of cattle used in study

Location	Hereford	*Bos taurus*	*Bos indicus*	Total
Texas Agrilife Research, College Station	4	73	5	82
Texas Agrilife Research, McGregor	1	2	81	84
Texas Agrilife Research, Overton			102	102
Oklahoma State University, Stillwater		102		102
University of Arkansas, Fayetteville		35		35
Louisiana State University, Baton Rouge			107	107
Mississippi State University, Starkville	29	40		69
Mississippi State University, Brown Loam Station, Raymond	1	24	17	42
University of Florida, Range Cattle Research and Education Center, Ona			90	90
University of Florida, North Florida Research and Education Center, Marianna			51	51
Clemson University, Clemson, SC	68	108		176
North Carolina State University, Tidewater Station, Plymouth		71		71
Texas A&M University, Commerce	17	43		60
J. Cloud Cattle, Rule, TX		12		12
Total	**120**	**510**	**453**	**1,083**

Other cameras used included Kodak CX7530 Zoom Digital Camera (Eastman Kodak Company, Rochester, NY) at the Brown Loam location and Canon EOS Rebel T3i (Canon Company, Ota, Tokyo, Japan) at the North Carolina location. Use of the camera flash was avoided; however in few cases where lighting was poor it was accepted. Examples of images taken at Texas Agrilife Research Center, McGregor, are shown in Figure 5.

Traits evaluated from these images included total eyelid pigmentation percentage, total corneoscleral pigmentation percentage, eyelid pigmentation percentage for upper and lower eyelids individually, and lesion presence or absence (coded as 1 or 0, respectively) as well as noting location of lesion as either E, eyelid or C, corneoscleral/eyeball.

Figure 5. Examples of images. Top--Full representation of pigment upper and lower eyelids visible in one image; Middle--Varying corneoscleral and eyelid pigmentation; photo taken angled up, in order to see under thick eyelashes; Bottom-- Varying eyelid pigmentation; photo taken straight on, in order to see both eyelids

Pigmentation quantification

Previous methods to analyze eyelid and corneoscleral pigmentation have included visual appraisal and scoring by laying a grid over an image of the eye and counting the area the pigment covered (Anderson et al., 1957; Anderson, 1960, 1963, 1991). Technology was adapted from work done with archaeological measuring tools. Nearly identical images for every eye are impossible with handling livestock, even when restrained in a chute. Therefore extensive editing and property manipulation of images were required before analysis. Various software packages were considered for manipulation and analysis of images.

The software Adobe Photoshop Elements 2.0 (Adobe Systems Incorporated, San Jose, CA) was used for manipulation of images due to their lack of uniformity. This included cropping the image to only the areas of interest- in one copy the eyelid region only and in another, the corneoscleral region only. Re-coloration of the images was done on an as needed basis with the “paint” tool and color was determined by the “dropper” tool. Re-coloration was only necessary if a shadow, eyelashes, or other non-cattle related obstruction hindered the true color of the eyelid or corneoscleral/eyeball regions. After the images were cropped and re-colored (if necessary) the images were converted to an 8-bit image (pixel intensity, 0-255) using the “Mode: Grayscale” function. It was necessary for the images to be in grayscale (8-bit) so that the pixel intensities, color of the individual pixel, were in whole numbers, 0-255. Figure 6 is an example of the program and image manipulation.

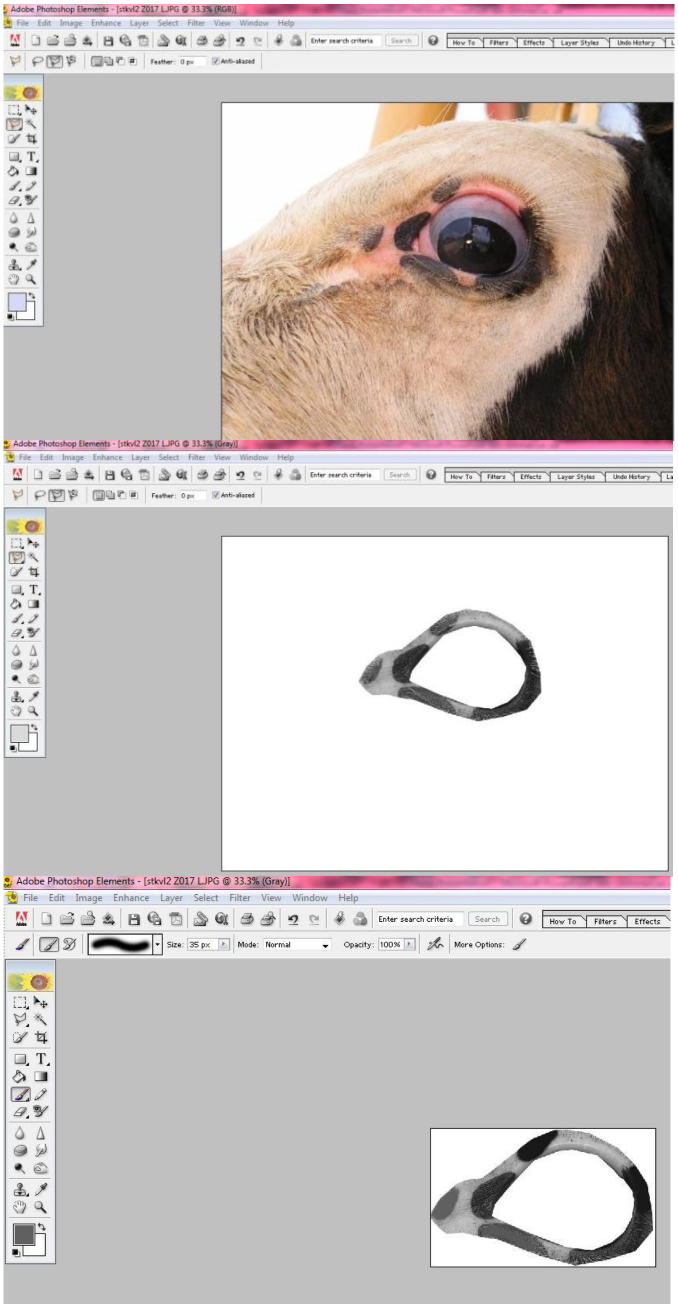

Figure 6. Example of image manipulation and progression in Adobe Photoshop 2.0

Image J software (Java, Bethesda, MD) was used via the "threshold" and selection tools to leave the eyelid and corneoscleral areas on a pure white (pixel intensity 255) background. The image was then saved as only its x and y coordinates for non-255 pixels, representing each pixel and its intensity (0-255).

Microsoft Excel (Microscoft Corporation, Redmond, WA) with extensive equations and addition of macros was used to quantify the percentage of pigment. This included upper eyelid percentage, lower eyelid percentage, a total eyelid percentage, as well as corneoscleral pigment percentage. A histogram for each image (separate image versions for eyelid and corneoscleral regions) was generated and a best-fit line graph was created with these points. The line graph represented the pixel intensity as the x axis and the number of pixels with specific intensity as the y axis. Loess regression of the x and y coordinates created a bimodal line over these points which determined primarily two peaks and a trough in the data. This used an average of the varying slopes in the best-fit line and determined where the averaged slopes changed sign, essentially, the first and second derivative of the line. Loess regression has a rigidity feature as to how well the line fits over the points; this had to be refined minimally on a limited basis to better delineate the two peaks and trough. These two peaks, or derivatives, represented the two groups of pixel intensities that were highest, essentially a pigmented (dark pigment) peak and an unpigmented (light pigment) peak. The trough indicated where to divide the pixel intensities into number of dark pixels and number of light pixels. Using these counts, a proportion of pigment was generated. Subsequent to calculating a total pigment proportion, the x and y coordinates of eyelid images were rotated objectively to separate the upper and lower eyelid.   This rotation was created by calculating a covariance matrix and an eigen-decomposition. The image coordinates was rotated to the major principle component axis, which was always along the long dimensions of the eyelid, then oriented over the x axis, effectively separating the upper and lower eyelid areas as pixels with positive and negative y values, respectively. The appropriate angle at which to rotate the image was determined to be about 43 degrees for most images. The pixel intensities per lid were counted using formulas in Excel, giving an upper eyelid proportion and a lower eyelid proportion of pigment. Figure 7 is an example of the program and quantitative analysis.

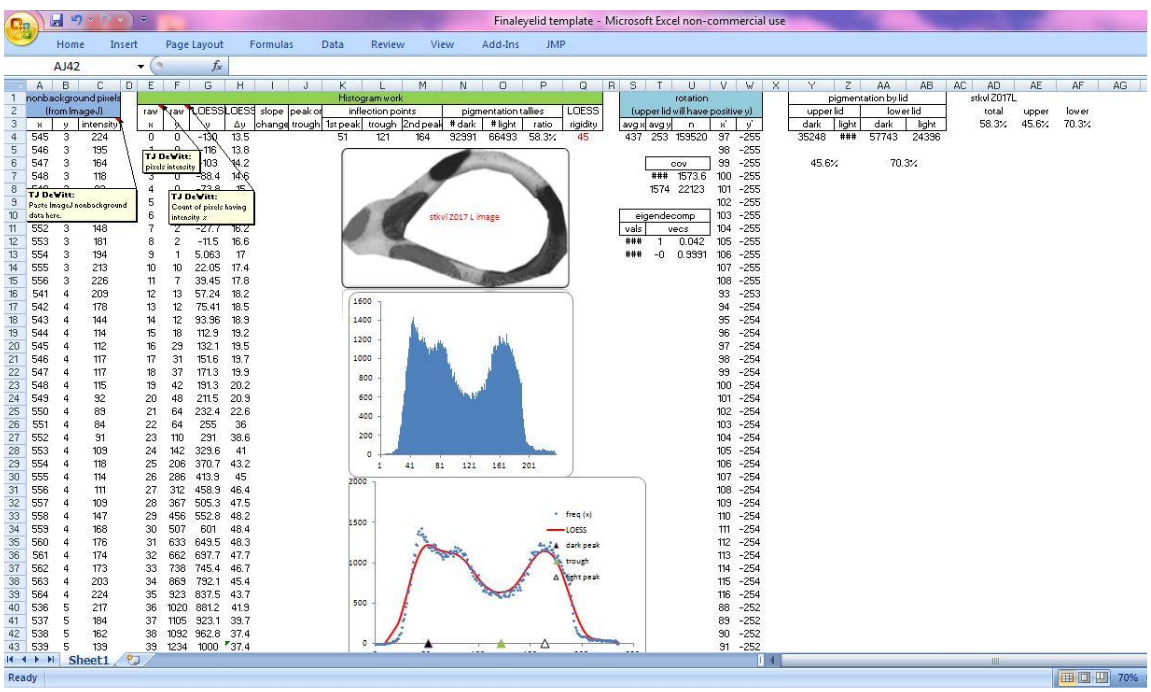

Figure 7. Microsoft Excel spreadsheet with image information and calculations

Statistical analysis

Traits were analyzed using linear models with ASReml 3 (Gilmour et al., 2009). Fixed effects investigated included breed type (Hereford, *Bos taurus* crosses, *Bos indicus* crosses), sex of animal (male, female, or undetermined), and age category (2 levels: cows 2 yr or older, and animals 1 yr or younger, including calves). Lesion presence or absence (denoted 1 or 0, respectively) was analyzed assuming a binomial distribution. A logit link function was applied to values for analyses and comparison of means; however, an inverse link function was applied to results before presentation. Pairwise comparisons of least squares means were conducted using *t* tests.

Data were also analyzed using a fully parametric test, MANOVA using JMP Pro 10 (SAS Inst., Inc., Cary, NC) with pigment percentages for all evaluated areas as the dependent variables. Fixed effects included breed type, sex, age category, and percentages of eyelid and corneoscleral pigment. A scatterplot matrix of pairwise correlations ($P < 0.01$) was used to visually assess associations. Permutation analysis was used to confirm the probability values, since test data were not normally distributed. A hierarchical approach to model selection was used to define the simplest model to represent effects. First a model was run with the independent variable interactions that could be tested. Not all interactions could be tested because some combinations of the main effects did not have replication. For example, there were no yearling male Herefords with lesions. Interactions that were not significant or marginally significant (i.e., $P \approx 0.05$) were removed from our model, beginning with the higher order interactions. Once the strong predictors were selected we reduced from six dependent variables (left and right, upper and lower eyelids and corneoscleral pigment measures) to left and right eyelid (i.e. upper and lower lids pooled) and corneoscleral pigment, and then to pooling left and right traits (i.e. to two dependent variables: eyelid and corneoscleral pigment. Significance structure was assessed and the simplest model indicating the same basic effect was selected as best.

RESULTS AND DISCUSSION

The described procedures for quantification of images successfully produced objective results for measuring eyelid and corneoscleral pigmentation.

Upper and lower eyelid pigmentation proportions were highly correlated (0.9 for both eyelids). Overall left and right eyelid pigmentation amounts were correlated (average: 0.65) and corneoscleral pigmentation was relatively independent from eyelid pigmentation (0.26), though all correlations are significant with this high sample size (Table 4). *P*-values for least squares means of multivariate analysis are listed in Table 5 with permutation analysis *P*-values listed concurrently. Although fixed effects were not normally distributed, it was important to recognize the relative differences between parameters rather than the individual means.

Table 4. Estimates of correlation coefficients for pairs of phenotypic variables from multivariate scatterplot analysis[1]

Pigment Correlations		**Left eyelid**		**Right eyelid**	
		Upper	Lower	Upper	Lower
Left	Upper eyelid		0.895	0.649	0.638
	lower eyelid			0.652	0.658
Right	upper eyelid				0.893
	lower eyelid				
		Total eyelid			
Total corneoscleral		0.230			

[1]All correlation coefficients differed from 0 ($P < 0.05$)

Table 5. P-values for least squares means from multivariate analysis of variance

	Actual	Permutation
Lesion effect	$P < 0.05$	$P < 0.05$
Breed effect	$P < 0.05$	$P < 0.05$
Age effect	$P < 0.05$	$P < 0.05$
Sex effect	$P = 0.73$	$P = 0.73$

Eyelid pigmentation

Neither sex nor age group significantly influenced eyelid pigmentation, they did not meet requirements for remaining in the final model (sex $P = 0.63$) (age $P = 0.26$). Hereford × *Bos taurus* or Hereford × *Bos indicus* had greater ($P < 0.05$) means for left upper and lower eyelid pigment proportions than did straight Herefords (Table 6). Right upper eyelid and right lower eyelid pigmentation proportions differed for all three breed types ($P < 0.05$). Multivariate analysis of variance results indicated that Hereford eyelid pigmentation proportions differed from Hereford × *Bos indicus* or Hereford × *Bos taurus* cross animals (Figure 8, 9, 10). Results indicated a difference between the Hereford breed and their cross bred counterparts for left and right eyelid pigmentation including upper and lower eyelid. Left eyelid pigmentation proportion means for Hereford × *Bos indicus* and Hereford × *Bos taurus* were not significantly different but Hereford × *Bos indicus* crosses had greater proportion of right eyelid pigmentation. It may be that this is an inherent breed type difference; it may also be that *Bos indicus*-Hereford composites (Brafords) and crosses have been more consistently selected for eyelid pigmentation.

Table 6. Breed type means for proportion of eyelid pigmentation by eye and eyelid of cattle with white faces, $n= 1083$[1].

Eye	Left		Right	
Eyelid	Upper	Lower	Upper	Lower
Breed type				
Bos indicus	0.94 ± 0.011^{a}	0.94 ± 0.010^{a}	0.94 ± 0.011^{a}	0.94 ± 0.011^{a}
Bos taurus	0.91 ± 0.011^{a}	0.92 ± 0.010^{a}	0.89 ± 0.011^{b}	0.90 ± 0.010^{b}
Hereford	0.65 ± 0.022^{b}	0.69 ± 0.020^{b}	0.68 ± 0.022^{c}	0.68 ± 0.021^{c}

[a-c] Within a column (trait), means that do not share a common superscript differ ($P < 0.05$).

[1]Means were generated from a linear model using ASReml (Gilmour et al., 2009).

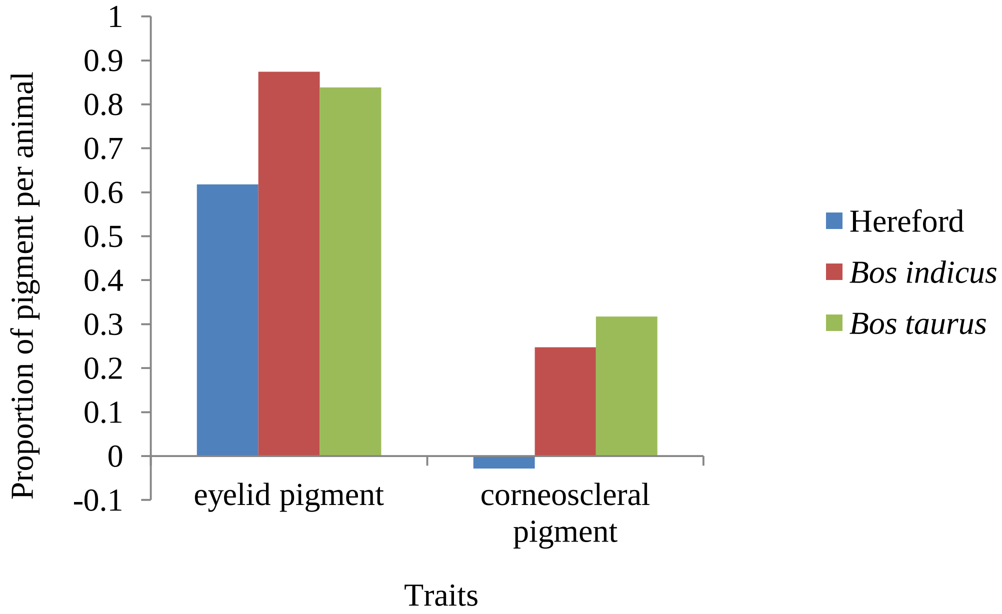

Figure 8. Least squares means for breed type by total eyelid and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

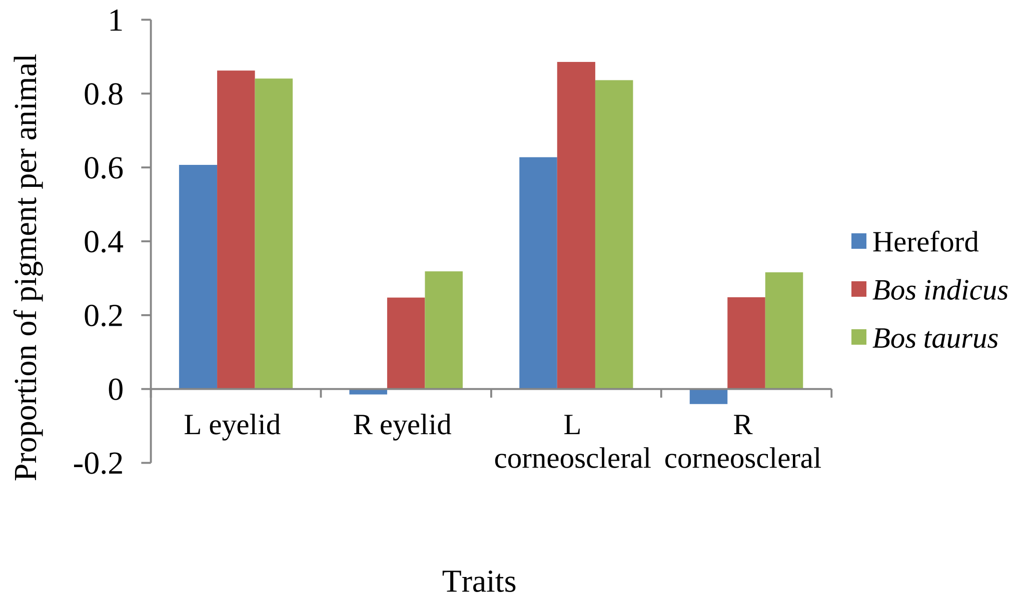

Figure 9. Least squares means for breed type by left (L) and right (R) eyelid and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

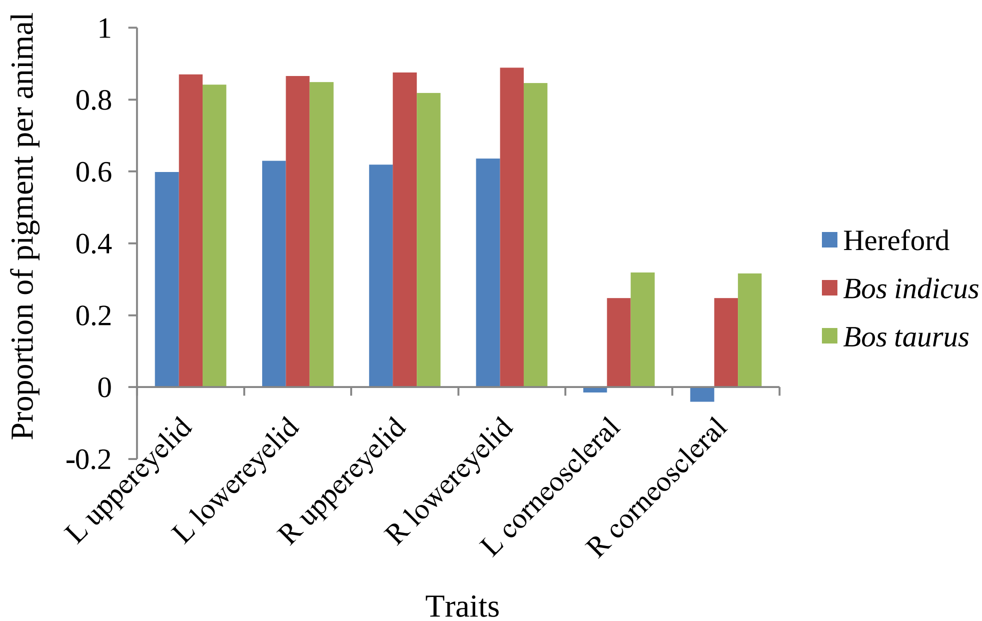

Figure 10. Least squares means for breed type by expanded factors left (L) and right (R), upper and lower eyelid and corneoscleral pigment proportions. Multivariate analysis of variance (P < 0.001)

Corneoscleral pigmentation

There were no sex differences detected for amount of corneoscleral pigmentation (P = 0.22 and P = 0.66 left and right respectively). Corneoscleral pigmentation proportions in each eye was largest (P < 0.05; Table 7) for *Bos taurus* crosses, intermediate for *Bos indicus* crosses, and lowest for Hereford. Corneoscleral pigmentation proportions in each eye differed by age category. Results from multivariate analysis of variance showed mature animals had more corneoscleral pigment than did the calves or yearlings (Figure 11, 12, 13). This was not surprising, as it has been earlier reported that corneoscleral pigmentation increases with age (Vogt et al., 1963; Anderson, 1991). A relationship between eyelid pigmentation and corneoscleral pigmentation was detected (r=0.26, Table 4), which was consistent with earlier reports of phenotypic correlations (Anderson, 1991; Vogt et al., 1963). Vogt et al., (1963) concluded low phenotypic correlations, but high genetic correlations.

Table 7. Breed type and age category means for proportion of corneoscleral pigmentation by eye of cattle with white faces, n=1083[1]

	Corneoscleral pigment	
Eye	Left	Right
Breed type		
Bos indicus	0.32 ± 0.017^{b}	0.32 ± 0.018^{b}
Bos taurus	0.39 ± 0.016^{a}	0.41 ± 0.016^{a}
Hereford	0.05 ± 0.034^{c}	0.04 ± 0.034^{c}
Age categories		
Calves and yearlings	0.16 ± 0.018^{b}	0.18 ± 0.018^{b}
Mature	0.34 ± 0.017^{a}	0.34 ± 0.017^{a}

[a-c] Within a column (trait), means that do not share a common superscript differ ($P < 0.05$).

[1]Means were generated from a linear model using ASReml (Gilmour et al., 2009).

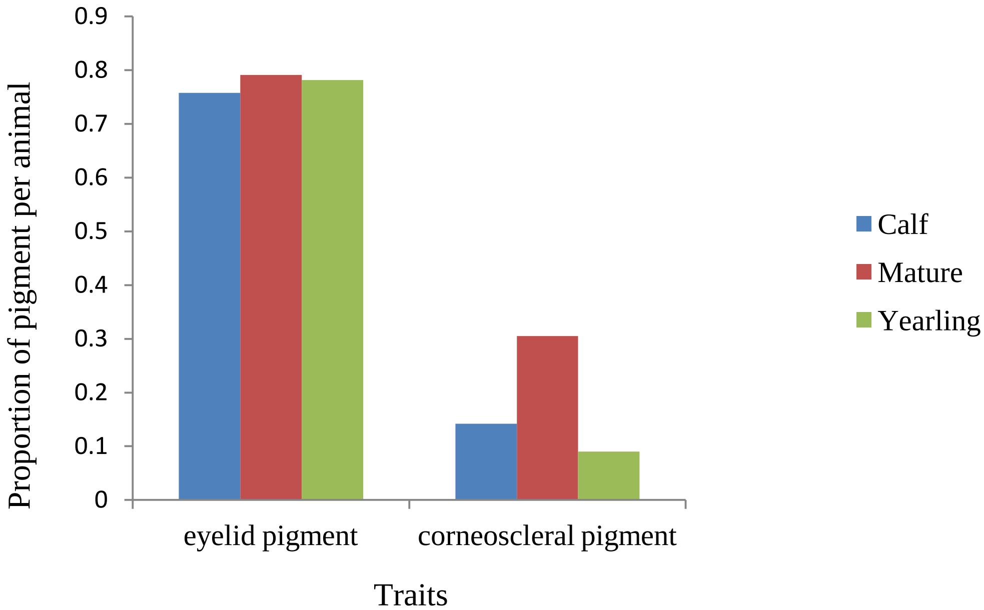

Figure 11. Least squares means for age categories by total eyelid and total corneoscleral pigment proportions. Multivariate analysis of variance (P < 0.001)

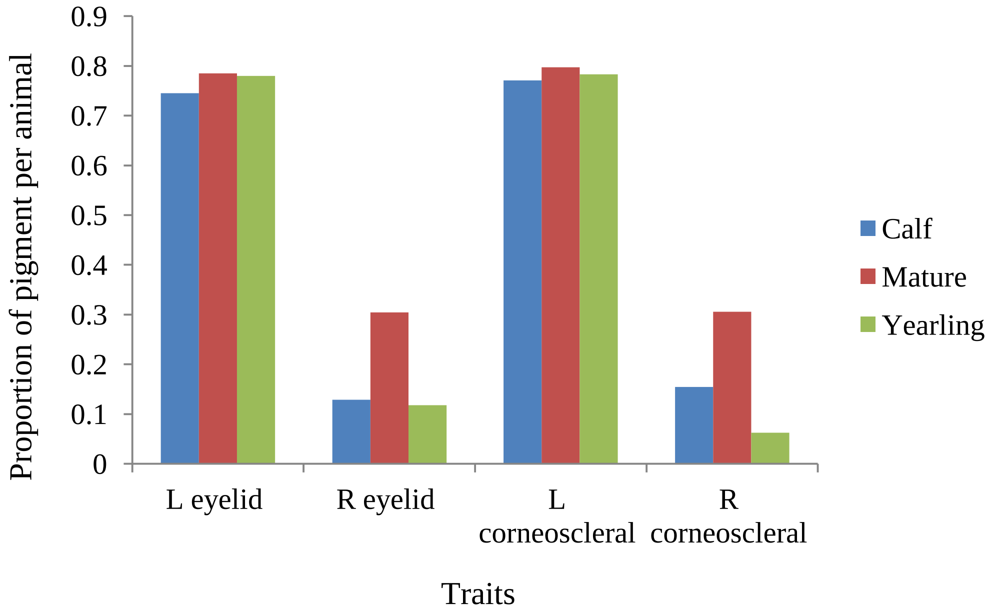

Figure 12. Least squares means for age categories by left (L) and right (R) eyelid pigment and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

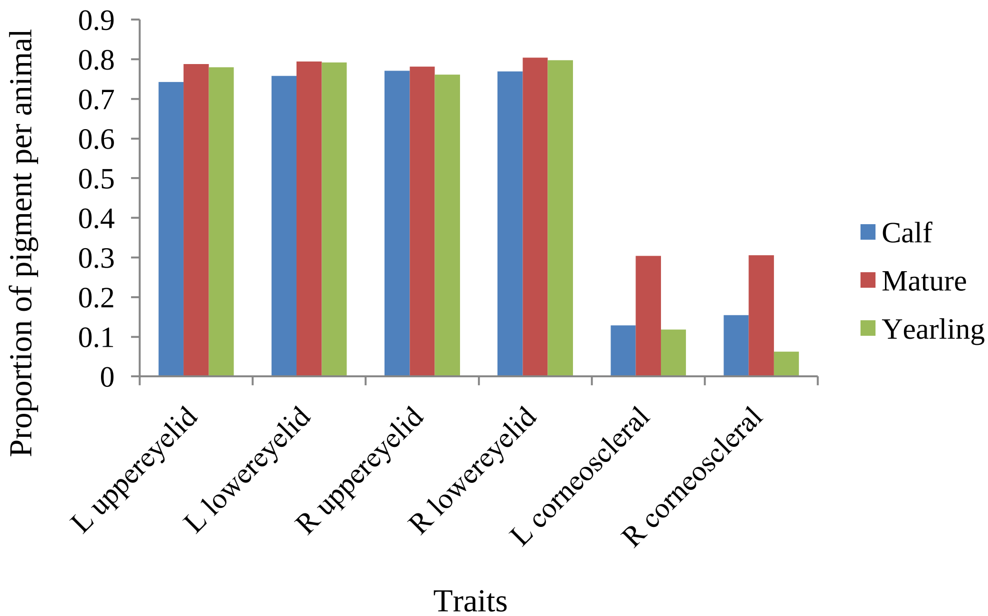

Figure 13. Least squares means for age categories by expanded factors left (L) and right (R), upper and lower eyelid and corneoscleral pigment proportions). Multivariate analysis of variance ($P < 0.001$)

Lesion presence or absence

Lesion presence was confirmed for 34 of the 1083 animals. Table 5 lists the animals with lesions by breed type, age category, location of the lesion and pigmentation status.

Table 8. Distribution of observed lesions across age category, breed type, location, and pigmentation

	Hereford	*Bos indicus*	*Bos taurus*
Age category			
Calf			2
Mature	19	8	5
Location			
Eyelid	9	3	6
Eyeball	7	5	1
Both	3		
Pigmented area?			
Yes	1	3	1
No	19	4	6

No sex differences were detected ($P = 0.8$) for any trait. Hereford × *Bos indicus* and Hereford × *Bos taurus* had lower proportion of lesions in either eye than Hereford animals ($P < 0.05$; Table 9). Young animals (calves and yearlings) had lower proportion of lesions than mature cattle (*P* < 0.05; Table 9). Only 2 of the 34 animals with lesions were young animals (Table 8). This result was consistent with earlier work (Woodward and Knapp, 1950), and was likely due to the greater quantity of exposure to harsh environmental conditions (particularly sun) across time. Results from multivariate analysis of variance indicated that cattle with lesions had overall less eyelid and corneoscleral pigment than those without lesions (Figure 14, 15, 16). This supports the earlier work with respect to eyelid pigmentation (Anderson, 1960), and importantly, represents the first confirmation of that association with respect to corneoscleral pigmentation, which was asserted by Vogt et al. (1963). Vogt et al. (1963) concluded increased corneoscleral pigment resulted in decreased lesion presence, and Anderson (1960) concluded that increased eyelid pigment resulted in decreased lesion presence.

Table 9. Breed type and age category means for proportion of lesions in cattle with white faces, $n = 34$[1]

	Lesion presence
Breed type	
Bos indicus	0.01 ± 0.005^{b}
Bos taurus	0.01 ± 0.003^{b}
Hereford	0.07 ± 0.022^{a}
Age categories	
Calves and yearlings	0.01 ± 0.002^{b}
Mature	0.06 ± 0.011^{a}

[a-b] Within a column (trait), means that do not share a common superscript differ ($P < 0.05$).

[1]Means were generated from a linear model using ASReml (Gilmour et al., 2009).

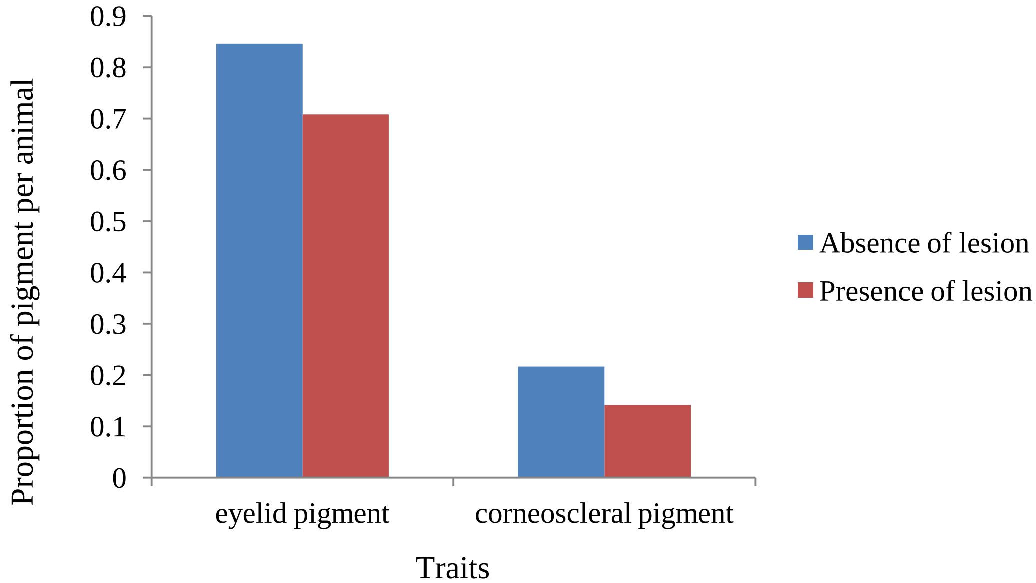

Figure 14. Least squares means for lesion presence or absence by total eyelid and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

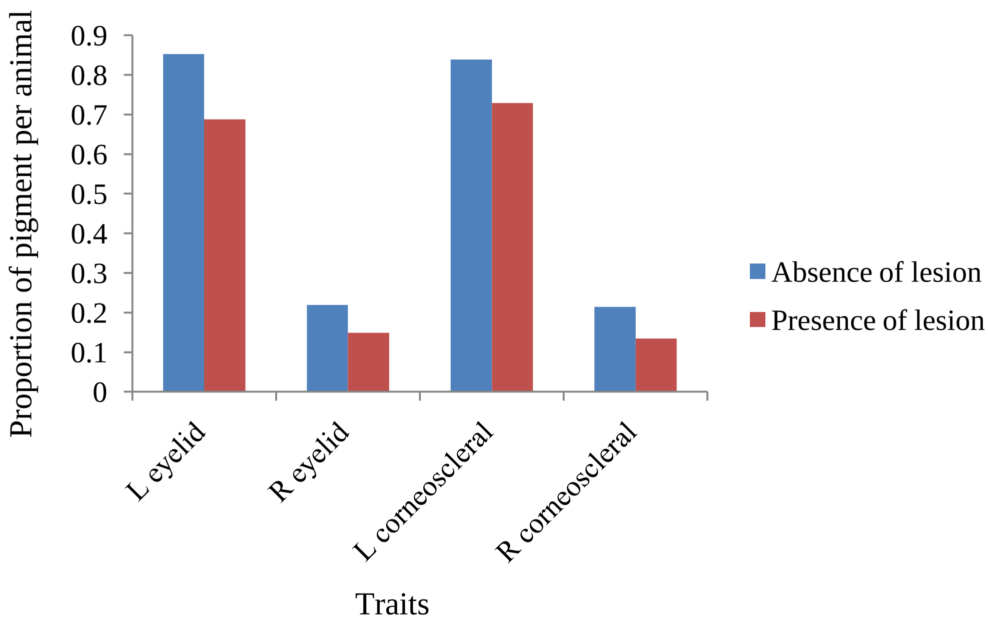

Figure 15. Least squares means for lesion presence or absence by left (L) and right (R) eyelid and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

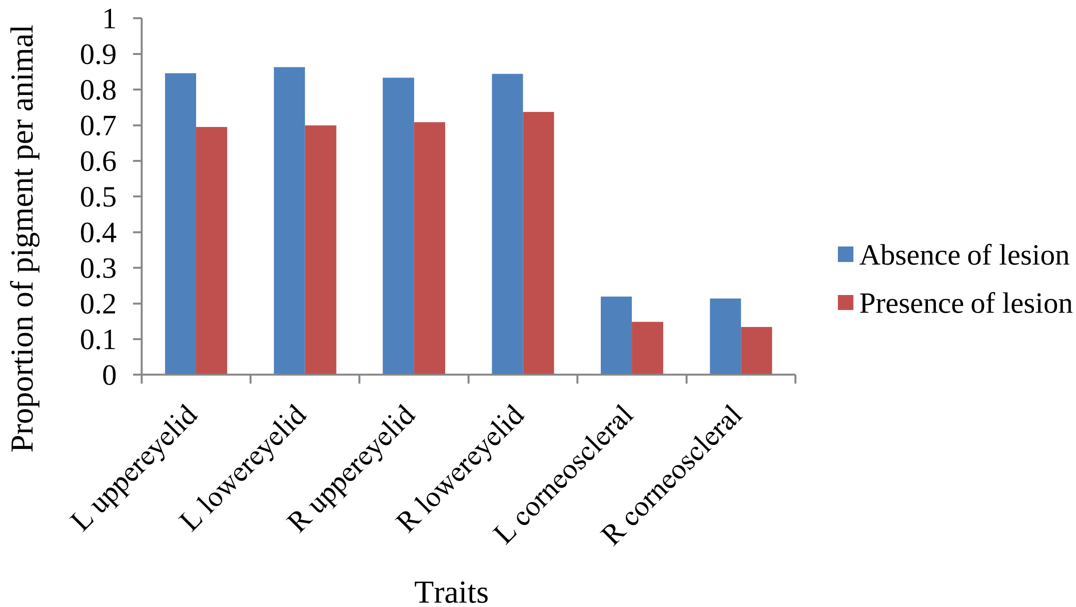

Figure 16. Least squares means for lesion presence or absence by expanded factors left (L) and right (R), upper and lower eyelid pigment and corneoscleral pigment proportions. Multivariate analysis of variance ($P < 0.001$)

There was potential confounding of breed type and age of animals with locations. It was not possible to separate the effect of location from breed type. *Bos indicus*-influenced animals were only represented in records from the 4 southernmost research locations. Variation among locations could indicate differential solar and ambient influence on these traits. It is likely that the populations sampled in this study were selected, by crossbreeding, to some degree for eye pigmentation. It is also likely that the proportion of lesions observed underestimates the overall proportion of lesions across time, as all locations cull animals with severe eye cancer or lesions.

CONCLUSION

This work represents updated assessment of pigmentation in white faced cattle using today's technology, including, obtaining digital images and objective image manipulation by quantification of pixels; previous work was based around subjective quantifications of amount of eyelid pigment. Consistent with earlier work, there was lower incidence of precancerous lesions in cattle with white faces with increased pigmentation around the eyes. Mature animals had greater proportion of corneoscleral pigmentation than young animals, and this is confirmation of earlier work as well. Monitoring individual animals across years would be a useful strategy to confirm that corneoscleral pigmentation increases with age. There were clear breed type differences in pigmentation of eyelids and corneoscleral pigmentation; least squares means indicated *Bos indicus* cross cattle had the most eyelid pigment and *Bos taurus* cross cattle had the most corneoscleral pigment. Importantly, crossbreeding Hereford with other breeds may increase pigmentation and thereby reduce the probability of cancer in the eye. Augmenting the numbers of purebred Herefords with records would permit better assessment of variation of these traits within the breed. Identification of genomic regions responsible for this trait variation could prove useful in selection programs.

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