Genome-wide association study of UV-protectant ambilateral circumocular pigmentation in Hereford cattle.

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Abstract

Hereford cattle have a dominantly inherited white face that is characteristic of their breed. A small proportion of Hereford cattle present with regions of pigmentation around the eyes, known as ambilateral circumocular pigmentation (ACOP), which is hypothesised to provide UV-protectant properties to the underlying tissue. ACOP has been associated with reduced incidence of two diseases, bovine ocular squamous cell carcinoma, and bovine infectious keratoconjunctivitis, making it a desirable trait for selection. We conducted a genome-wide association study for ACOP in 605 American Hereford cattle using 43,789 genetic markers, and investigated the phenotypic association between face-colour and ACOP. The most highly associated variant from our genome-wide association study mapped to Chr6 g.71059814G>A and explains most of the variation for this trait. This variant falls in close proximity to the well-known coat-colour patterning gene *KIT*, suggesting it may be the causal gene for this trait. Our results suggest that ACOP is not directly influenced by face-colour, and may be influenced by non-additive genetic effects. These results provide an opportunity to select for Hereford cattle with ACOP, enhancing welfare, and reducing the incidence of bovine infectious keratoconjunctivitis and bovine ocular squamous-cell carcinoma.

Keywords: Hereford cattle; ambilateral circumocular pigmentation; ocular squamous cell carcinoma; infectious kerato-conjunctivitis; GWAS; pigmentation traits

Introduction

Hereford cattle are a popular beef breed easily identifiable by their dominantly inherited white face, a striking trait actively selected for by pedigree breeders as an indicator of 'true breeding'. The white-face trait manifests due to a complete lack of pigmentation (melanin), attributable to the lack of functional pigment cells (melanocytes) occupying the skin and hair within the face region. Melanin functions as an antioxidant and radical scavenger to protect the skin and underlying tissues against damage from ultraviolet (UV) radiation, so although the white-face trait is cosmetically attractive to breed enthusiasts, the lack of eyelid pigmentation increases the risk of damage from the sun and at least two diseases: ocular squamous cell carcinoma (OSCC), and bovine infectious keratoconjunctivitis (BIK; Heeney & Valli 1985; D'Mello et al. 2016; Seid 2019). The most common form of malignant tumour affecting cattle is OSCC which occurs on the eye and eyelids. This disease occurs at a high frequency in Hereford cattle (Heeney & Valli 1985). The occurrence of OSCC causes reduced longevity and economic loss as cancer-stricken cattle are usually rejected, or condemned, at the meat-processing plant as unfit for human consumption. Russell et al. (1976), followed 396 Hereford cows over five years and observed that 51% of these cows were identified to have a lesion on their eye at least once in their life, and White & Moore (2009) reported that OSCC accounted for approximately 11.6% of the beef-carcass condemnations between 2003 to 2007.

Some white-faced Hereford, Simmental and Fleckvieh cattle have regions of pigmentation around the eyes known as ambilateral (both sides) circumocular (around the eyes) pigmentation. In Fleckvieh cattle, the frequency of ambilateral circumocular pigmentation (ACOP) is estimated to be around 36% (Mészáros et al. 2015), and in Hereford cattle it is suggested that approximately two thirds of the population have at least some pigmentation around the eyes or on the eyelids (Anderson et al. 1957). ACOP is associated with reduced incidence of OSCC and BIK in white-faced cattle (Ward & Nielson 1976; Davis 2013). ACOP has a reported heritability of between 0.41 and 0.50 (Anderson et al. 1957), indicating that selection for this trait in white-faced cattle would increase the occurrence of ACOP and would likely reduce the incidence of disease. Although the heritability of ACOP and its correlation with OSCC and BIK have been extensively studied, to our knowledge only one study has investigated the genetics of this trait. Pausch et al. (2012) estimated breeding values for ACOP in a Fleckvieh cattle population of 320,186 cows and 3,579 genotyped bulls. That study reported twelve quantitative trait loci (QTL) associated with ACOP across chromosomes 2, 5, 6, 11, 13, 14 and 22, and implicated well-established pigmentation genes *paired box 3* (*PAX3*), *proto-oncogenne receptor tyrosine kinase* (*KIT*), and *micropthalmia-associated transcription factor* (*MITF*), among others. They also reported higher heritability values for ACOP (0.79±0.04) than previously reported by Anderson et al. (1957).

The aim of our study was to investigate the genetic architecture of ACOP in Hereford cattle. We report a single significant signal on chromosome 6, and suggest *KIT* as the likely causal gene at this locus, with a single nucleotide polymorphism (SNP)-based heritability of 0.75. The results reported in this paper may inform future breeding decisions to select for ACOP in Hereford cattle, thereby enhancing welfare and profitability, especially in pasture-based farming systems where cattle are directly exposed to potentially damaging UV radiation.

Materials and methods

Study population

Face colour (white, splotchy or speckled) and the presence of pigmentation around the eye (one eye, two eyes, or none) were visually scored from photographs of 706 Hereford cattle taken in August of 2019. The cattle were sired by 102 bulls in a progeny test programme, with 38 bulls having more than five offspring within the dataset. The cattle were raised on the Olsen Hereford Ranch in Harrisburg, Nebraska, USA. DNA samples from ear tissue biopsies were extracted and genotyped by GeneSeek (Lincoln, NE, USA) using an Illumina Bovine 50k SNP Chip. All SNP positions used and mentioned in this study are based on the ARS-UCD1.2 bovine genome build (Rosen et al. 2020).

Population structure adjustments and GWAS

A genomic relationship matrix (GRM) was generated using 50k SNP Chip genotype data for each bovine autosome in GCTA (v1.93.2beta) to address possible population stratification in the association model due to relatedness. We treated the ocular pigmentation trait as a binary variable and used a 'leave one chromosome out' approach to conduct a mixed-linear-model-based association analysis in GCTA, which incorporated the GRM as previously described by Jivanji et al. (2019). We investigated the potential functional impact of all variants that surpassed the Bonferroni-corrected genome-wide significance threshold ($P=5\times10^{-8}$) using the Ensembl Variant Effect Predictor (VEP; McLaren et al. 2016). In subsequent analyses, we fitted individual face-colour classes (white face, splotchy face, and speckled face), and the tag SNP genotype as separate covariates in the model.

Mode of inheritance and trait heritability

Dominant and recessive modes of inheritance for the ACOP trait were tested on the most significant signal identified in the GWAS (chromosome 6) by using the '--model' function in PLINK (v1.9; Purcell et al. 2007). In the dominant and recessive models, the minor allele (in this example the G allele) was used as the reference allele to define recessive and dominant encoded genotypes. The dominant model tested the effect of GG and AG versus AA, the recessive model tested the effect of GG versus AG and AA, and the additive model tested the regression of the number of G alleles on the presence or absence of ACOP. The ACOP trait was treated as a binary variable, as described above, and the association model was conducted on genetic markers across chromosome 6 (n=2,102). A principal-component-analysis-based approach was used to account for stratification due to relatedness in this model. Eigenvectors were calculated using all 50k SNP Chip markers, and the top ten principal components were fitted as covariates, based on visualisation of principal component clusters and a scree plot of the eigenvalues. The top ten principal components explain more than 90% of the genotype variance in our population. A genome-wide GRM was used to estimate the SNP-based trait heritability in GCTA-GREML (v1.93.2beta), where the trait prevalence was set to 0.34 based on a study by Mészáros et al. (2015).

Results

Genome-wide association analysis

Phenotypes of the Hereford population comprised 101 cattle with pigmentation around just one eye (unilateral circumocular pigmentation; UCOP), 240 cattle with pigmentation around both eyes (i.e., ACOP), and 365 cattle with no pigmentation around their eyes. The genome-wide association study (GWAS) for the absence or presence of any eye pigmentation (UCOP or ACOP; n=706 cattle), and that for the presence or absence of ACOP (n=605 cattle), both revealed the same signal. The results for both GWAS revealed a single signal on chromosome 6 that exceeded our multiple-hypothesis-testing threshold ($P=5\times10^{-8}$), and mapped to the same top variant at Chr6 g.71059814G>A (UCOP or ACOP $P=5.2\times10^{-19}$; just ACOP $P=1.7\times10^{-22}$; Fig. 1). Since the P value was more significant for the second analysis, where cattle with ACOP were compared to cattle with no circumocular pigmentation, this phenotype became the focus of the analyses that are presented below.

Influence of face colour on ACOP

Within our population, we observed Hereford cattle to have one of three different face-colour classes: white face, splotchy face, or speckled face (Table 1), where UCOP or ACOP were not considered in the allocation of animals to these three classes. The occurrence of ACOP did not appear to segregate with any one face-colour class, appearing to manifest regardless of face colour. In our association model, we accounted for face-colour class and found that the top variant (Chr6 g.71059814G>A) remained at the top of the peak with very marginal shift in significance (white face $P=3.2\times10^{-22}$; splotchy face $P=2.7\times10^{-21}$; speckled face $P=1.2\times10^{-22}$).

Analysis of chromosome 6 locus

The top variant at the chromosome 6 locus was a SNP at Chr6 g.71059814G>A (rs134749374; $P=1.7\times10^{-22}$), which maps to intron 1 of the *Neuromedin-U* (*NMU*) gene, and is 805 kb downstream of the *proto-oncogene receptor tyrosine kinase* (*KIT*) gene. We detected 43 additional variants that exceeded the genome-wide wide significance threshold ($P=5\times10^{-8}$), spanning approximately 20 Mb in the vicinity of the top variant. Our investigation of all genes that mapped to this region identified the *KIT* gene as the most likely causal gene. Assessment of the predicted functional impact of the top (n=44) variants using Variant Effect Predictor (VEP) did not present any variants with obvious impacts on gene expression and/or protein function: one variant was predicted to be a 3' untranslated region variant, two variants were upstream gene variants, 11 variants were intron variants, and the remaining 30 variants were intergenic. Likewise, the top associated variant was not found to map to a highly conserved region that could

Figure 1 Manhattan plot based on genome-wide association analysis for the absence or presence of ambilateral circumocular pigmentation in Hereford cattle. There is a single signal that exceeds the significance threshold indicated by the dashed line ($P=5\times10^{-8}$) on chromosome 6, and the top variant maps to Chr6:71,059,814bp with a P value of 1.7×10^{-22}.

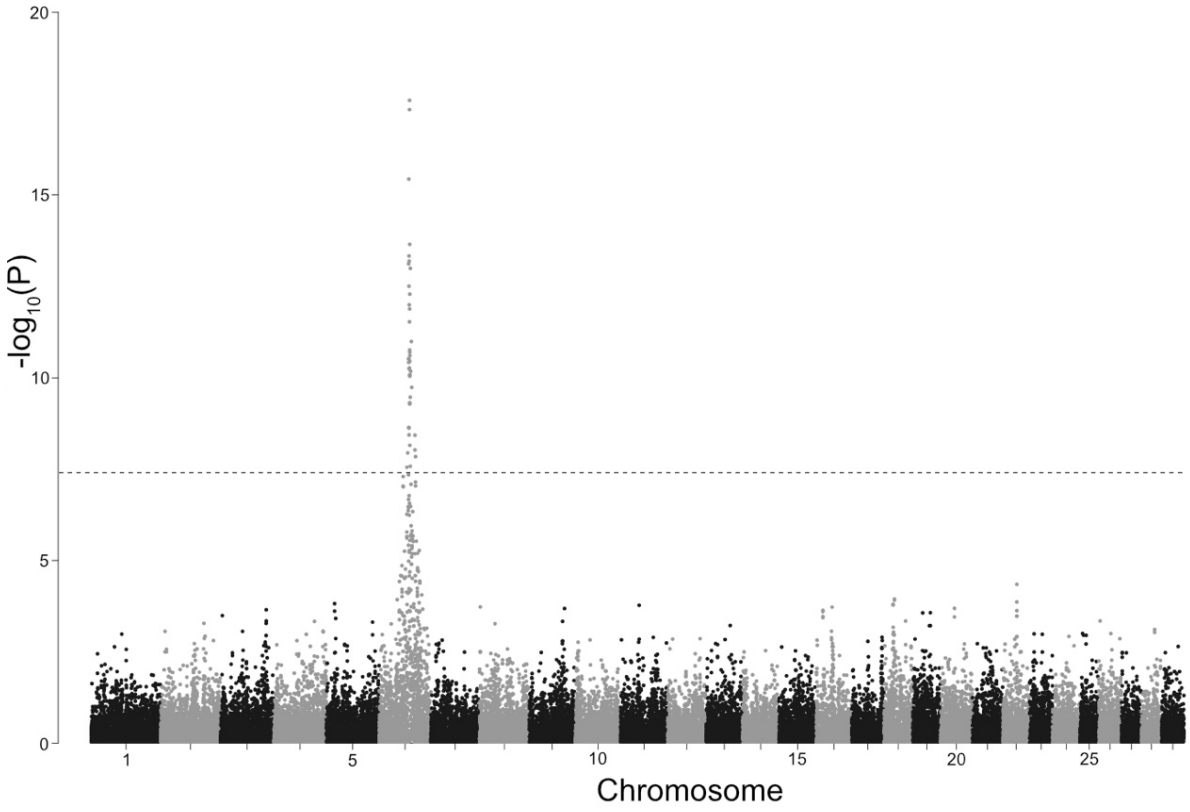


Table 1 Image of each trait scored and number of cattle scored as having no pigment around their eyes versus pigment around both eyes (ambilateral circumocular pigmentation) for each face colour trait: white face, splotchy face, and speckled face. Cattle with both splotchy faces and speckles (n=7) were counted twice.

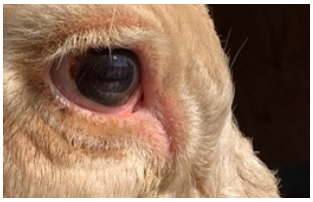
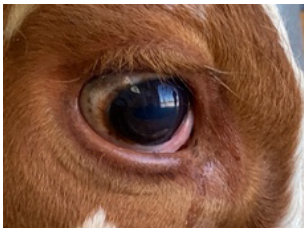
	White Face	Splotchy Face	Speckled Face
			
No pigment around the eyes 	332	2	28
Pigment around both eyes 	151	71	28

Table 2 Top 10 variants for the chromosome 6 locus detected in the genome-wide association analysis for the presence or absence of ambilateral circumocular pigmentation in Hereford cattle, with minor allele frequency, predicted variant effects from Ensembl Variant Effect Predictor, and gene the variant maps to.

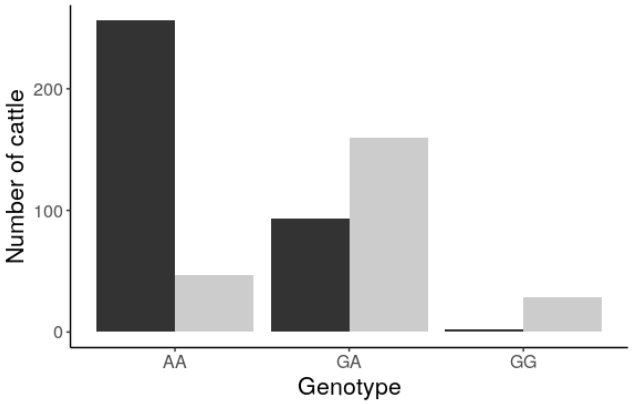
Variant reference ID	Genomic position on Chr6	P value	Minor allele frequency	Predicted variant effect	Gene symbol
rs134749374	g.71059814G>A	1.7×10^{-22}	0.27	Intron variant	*NMU*
rs135243955	g.69234941C>T	2.6×10^{-18}	0.19	Intergenic variant	-
rs110185900	g.69224236C>T	4.6×10^{-18}	0.19	Intergenic variant	-
rs109877370	g.67582129C>T	3.7×10^{-16}	0.22	Intergenic variant	-
rs110547897	g.69596878C>T	2.2×10^{-14}	0.27	Upstream gene variant	*CHIC2*
rs132937166	g.68028962T>C	4.7×10^{-14}	0.2	Intergenic variant	-
rs133388993	g.68428584G>A	6.5×10^{-14}	0.32	Intergenic variant	-
rs109242003	g.67159718C>T	7.7×10^{-14}	0.13	Intergenic variant	-
rs29017695	g.71028209G>T	1.0×10^{-13}	0.17	Upstream gene variant	*PDCL2*
rs41653781	g.67991798A>C	3.1×10^{-13}	0.27	Intergenic variant	-

support functional impact, as might be expected given the variants represented 50k SNP chip data only. Table 2 lists the top ten associated variants from chromosome 6 and their predicted functional effects. After fitting the top variant as a covariate in our association model, most of the significance was removed from the locus (smallest P value was 0.0005 for Chr6 g.27370287A>G), suggesting a single biallelic QTL as responsible.

Mode of inheritance

The model used to test the contribution of additive, dominant, and/or recessive inheritance on the manifestation of ACOP identified the same top SNP from the previous additive model conducted in GCTA: Chr6 g.71059814G>A. The smallest P value for this variant came from the additive model ($P=1.6\times10^{-37}$), followed by the dominant model ($P=2.0\times10^{-36}$), and then the recessive model ($P=4.8\times10^{-10}$). Although these results support an additive mode of inheritance, the small number of cattle with ACOP that were homozygous for the minor allele of this variant (n=29, minor allele frequency=0.27) may have limited our ability to resolve the mode of inheritance. When we examined the difference between phenotype (ACOP or no ACOP) by genotype class, we observed a larger difference in phenotype for the AA genotype, compared to the AG and GG genotypes. These results may allude to a non-additive mode of inheritance (Fig. 2). The heritability of ACOP estimated by GCTA-GREML was 0.75±0.07.

Figure 2 The number of cattle observed to have ambilateral circumocular pigmentation (grey) or no pigmentation around the eyes (black) by genotype class for tag variant Chr6 g.71059814G>A.



Discussion

To the best of our knowledge, we present the first genome-wide association analysis for ACOP in Hereford cattle. Using phenotype scores for 605 American Hereford cattle, and 43,789 genome-wide genetic markers, we identified a single significant association signal on chromosome 6, mapping to Chr6 g.71059814G>A ($P=1.7\times10^{-22}$). Unexpectedly, the strength of association for this signal was not affected by face colour, suggesting that ACOP is controlled by different alleles to those that influence the colour of the face in Hereford cattle. We suggest that the *KIT* gene is the most likely causal gene for ACOP, and given that the white-face trait has also been attributed to dominant mutations of the same gene (Whitacre 2014), these findings suggest multiple segregating pigmentation QTL in these animals.

There were 44 variants that exceeded the genome-wide significant threshold ($P=5\times10^{-8}$) on chromosome 6, with the most significant variant mapping 805 kb downstream of the *KIT* gene. Although this variant is nearly 1 Mb away from *KIT*, other variants with P values of a similar magnitude mapped within 240 kb of the *KIT* gene. Given the use of a relatively sparse genotyping platform (43k SNP) for mapping, and the status of *KIT* as one of the most-frequently implicated and best-characterised pigmentation genes in mammals, these findings suggest *KIT* as the likely causal gene for the ACOP trait. The KIT protein is a tyrosine kinase mast/stem cell growth factor receptor that is involved in melanocyte stem-cell migration out of the neural crest during embryonic development. KIT facilitates melanocyte migration along the neural crest to the final destination in the skin. Mutations that alter *KIT* expression or activity are hypothesised to impact stem-cell migration,

proliferation and/or survival. This impairs the ability of melanocyte precursors to colonise their final destination, causing regions of the skin and hair to lack pigmentation and appear white (Adameyko et al. 2009; D'Mello et al. 2016; Mort et al. 2016). Bioinformatic prediction of the significant GWAS variants for functional impacts did not detect any likely causal mutations. However, when the most highly associated SNP Chr6 g.71059814G>A, was fitted as a fixed effect in the association model, this SNP accounted for the majority of the signal at this locus. Although it is possible that Chr6 g.71059814G>A may have some effect on gene expression or regulation, this is unlikely as the variant does not appear to be highly conserved amongst vertebrates, and represents but one of many candidates that would likely be represented if GWAS was performed with higher-density genotype data (e.g., whole-genome sequence). More likely, Chr6 g.71059814G>A tags the causal effect for the manifestation of ACOP and is in linkage disequilibrium with the causal mutation. The ACOP trait has also been studied in Fleckvieh cattle, where Pausch et al. (2012), reported several significant signals across the genome. The most significant signal was reported on chromosome 6, mapping to Chr6 g.70362480T>C ($P=1.1\times10^{-11}$). That variant was not captured in our genotype data, but maps within 700 kb of our tag SNP and is likely tagging the same causal mutation. The heritability reported in our population (0.75±0.07), falls close to that reported by Pausch et al. (2012) (0.79±0.04), perhaps supporting a common genetic architecture. Future studies could use whole-genome sequencing of cattle with ACOP from both Fleckvieh and Hereford to fine-map the causal mutation for ACOP, and establish if ACOP is caused by the same genetic effect in both breeds.

The dominantly inherited white-face trait in Hereford cattle has also been mapped to chromosome 6, locating upstream of the *KIT* gene. The white-face trait manifests if cattle carry one or more copies of a large tandem duplication approximately 50 kb upstream of the *KIT* gene. The tandem duplication is hypothesised to impair *KIT* expression, preventing functional melanocytes from occupying the skin and hair of the face, resulting in the lack of pigmentation in this region (Whitacre 2014). As ACOP is superimposed on the white-face trait, we investigated if face-colour influenced the presence/absence of ACOP. Fitting each face colour class (white, splotchy and speckled) as covariates in the model did not affect the GWAS signal. These results suggest that face colour cannot account for the signal observed at the chromosome 6 locus, and the manifestation of ACOP is caused by a different allele, perhaps at the same locus. Due to the molecular mechanism proposed to cause the white-face trait, and its dominant mode of inheritance, we investigated the possibility of a non-additive mode of inheritance for ACOP. Comparison of cattle with and without the ACOP trait by genotype class possibly supports a non-additive mode of inheritance, although the small number of cattle with ACOP that were homozygous for the minor allele limited our ability to formally resolve mode of inheritance. An epistatic mode of inheritance would not be entirely surprising as the GWAS signal maps near the *KIT* gene, the causal gene for the white face trait. It is possible that while the white-face mutation impairs *KIT* expression, the ACOP mutation may have a positive effect on *KIT* expression and/or function, allowing functional melanocytes to migrate to, and occupy the eye area, pigmenting the surrounding skin and hair. These results are speculative and would require a larger population size to further investigate the mode of inheritance, and possible epistatic interaction between the white-face mutation and ACOP mutation.

Our results identify a strong association between Chr6 g.71059814G>A and the ACOP trait in American Hereford cattle, and implicate the likely involvement of the *KIT* gene. Our results also suggest genetic complexity with regard to the mode of inheritance, and we suggest that ACOP in Hereford cattle and Fleckvieh cattle may be caused by the same mutation, but more work would be required to support this. Selection for ACOP in white-faced cattle could enhance welfare and profitability, by reducing the incidence of OSCC and BIK, especially in cattle raised in pasture-based grazing systems. Although this work has been done in American Hereford cattle, ACOP has also been observed in New Zealand Hereford cattle, and future studies could aim to investigate if the same genetic variants influencing ACOP segregate in local populations. To our knowledge, New Zealand Hereford breeders do not actively select for ACOP, but may benefit from considering ACOP-associated genetic variants in their selection index. Future work may aim to investigate the causal mutation for ACOP in New Zealand Hereford populations and develop a gene test to select appropriate sires and increase the frequency of ACOP in future generations.

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