Association of MHCY genotypes in lines of chickens divergently selected for high or low antibody response to sheep red blood cells

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ABSTRACT The chicken MHCY region contains members of several gene families including a family of highly polymorphic MHC class I genes that are structurally distinct from their classical class I gene counterparts. Genetic variability at MHCY could impart variability in immune responses, but robust tests for whether or not this occurs have been lacking. Here we defined the MHCY genotypes present in 2 sets of chicken lines selected for high or low antibody response, the Virginia Tech (**VT**) HAS and LAS, and the Wageningen University (**WU**) HA and LA lines. Both sets were developed under long-term bidirectional selection for differences in antibody responses following immunization with the experimental antigen sheep red blood cells. Lines in which selection was relaxed (VT HAR and LAR) or lacking (WU C) provided controls. We looked for evidence of association between MHCY genotypes and antibody titers. Chickens were typed for MHCY using a recently developed method based on a multilocus short tandem repeat sequence found across MHCY haplotypes. Five MHCY haplotypes were found segregating in the VT HAS and LAS lines. One haplotype was present only in HAS chickens, and another was present only in LAS chickens with distribution of the remaining 3 haplotypes differing significantly between the lines. In the WU HA and LA lines, there was a similar MHCY asymmetry. The control populations lacked similar asymmetries. These observations support the likelihood of MHCY genetics affecting heritable antibody responses and provide a basis for further investigations into the role of MHCY region genes in guiding immune responses in chickens.

Key words: MHCY, heritable antibody responses, chicken, Virginia Tech HAS and LAS, Wageningen University HA and LA

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INTRODUCTION

Immune defense in vertebrates involves multiple steps. Many cell types and numerous cellular interactions contribute to immune responses against microorganisms that cause disease. Early steps are typically without antigen specificity. In later steps, class I and class II cell surface proteins encoded in the classical major histocompatibility complex (**MHC**) region guide adaptive (memory-based) immunity. These loci are typically polymorphic, closely grouped, and genetically linked within a single chromosomal region. Individuals in outbred populations often carry different alleles at these loci, thereby providing genetic variation thought to be adaptive for populations facing pathogen challenge.

In chickens, the classical MHC is called MHCB. It contains genes encoding class I and class II peptide-antigen presenting molecules, genes for antigen processing, and the polymorphic BG gene family (Miller and Taylor, 2016). Chickens, turkeys and pheasants have a second region of polymorphic MHC-like genes called MHCY (also known as *Rfp-Y* or MHC-*Y*) (Briles et al., 1993; Reed et al., 2011; Miller and Taylor, 2016; Wittzell et al., 1995). In chickens, MHCY and MHCB are located on the same arm of chromosome 16. Haplotypes at these 2 regions assort independently (Delany et al., 2009; Solinhac et al., 2010).

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MHCY is not simply a duplicate of the classic MHCB region in chickens. While clearly recognizable as a region containing MHC class I genes, the class I genes in MHCY are distinctly different from MHCB class I genes (Afanassieff et al., 2000; Afanassieff et al., 2001). As is typical of MHC class I molecules, MHCY has an alpha chain that assembles with beta2-microglobulin and folds to form an antigen binding groove (Afanassieff et al., 2001). The binding groove appears to be hydrophobic and too narrow to accommodate peptide antigens (Hee et al., 2009; Hee et al., 2010). The distribution of conserved and polymorphic MHCY class I residues is clearly different from the distribution of equivalent residues in classical MHC class I molecules (Afanassieff et al., 2001; Hee et al., 2010; Miller et al., unpublished data). The distinctive nature of MHCY class I is further supported by evidence from mass spectrometry that MHCY class I molecules bind lipid ligands (Miller et al., unpublished data). Other genes in MHCY include several MHCY class II beta-chain genes, numerous C-type lectin-like genes, *LENG9* genes, and zinc finger protein genes (Rogers et al., 2003; Miller et al., unpublished data). There is strong evidence that MHCY haplotypes vary in size with different haplotypes containing different numbers of the genes within these 5 gene families. There are many LINE/CR1 and LTR retro-elements within MHCY. All these features define MHCY as a polymorphic region clearly different from MHCB and as a gene region that should be further studied.

Whether and how the MHCY region contributes to immune responses in chickens is unknown. Until recently, testing for association between MHCY genotypes and phenotypic traits was difficult because MHCY genotyping involved time-consuming Southern hybridizations that revealed haplotype-specific restriction fragment patterns (**RFP**). Never-the-less, early tests with RFP-typed samples provide some evidence for a role of MHCY in engraftment of skin transplants (Pharr et al., 1996; Thoraval et al., 2003). Links between MHCY haplotypes and regression of Rous sarcoma virus-induced tumors were reported in several studies (LePage et al., 2000; Pinard-van der Laan et al., 2004; Praharaj et al., 2004). Association of MHCY with the incidence of Marek's disease was noted in one trial, but not others (Wakenell et al., 1996; Vallejo et al., 1997; Lakshmanan and Lamont, 1998). Gene expression studies show MHCY class I genes are broadly expressed (Afanassieff et al., 2001; Hunt et al., 2006) and are among genes that change in expression during immune responses (Connell et al., 2012; Geng et al., 2015; Wu et al., 2015; Deist et al., 2018). Overall, these studies suggest a contribution from MHCY to the genetics of immune responses. Now that large numbers of chickens can be MHCY genotyped easily (Zhang et al., 2020), more robust tests for MHCY encoded functions can be performed.

The aim of this work was to test for a potential link between MHCY genetics and antibody responses in 2 experimentally controlled populations where selection has resulted in immune adaptation (Bovenhuis et al., 2002; Lillie et al., 2017). In both populations, chicken lines were selected over multiple generations for divergent antibody responses to immunization with the experimental antigen, sheep red blood cells (**SRBC**). In both, lines emerged with distinct antibody responses. Primary in the current study were the Virginia Tech (**VT**) high antibody selected (**HAS**) and low antibody selected (**LAS**) lines. The HAS and LAS lines are continuously selected at each generation, now over more than 45 generations, for high or low antibody response to SRBC. Maintained in parallel by random mating are HAR and LAR lines in which selection has been relaxed after an initial phase of high or low antibody selection. Two chromosomal regions affecting immune functions have been found to differ between HAS and LAS lines. These include MHCB on GGA16, and a region on GGA2 containing *SEMA5A* (semaphorin 5A), and *TGFBR2* (transforming growth factor beta-receptor 2) genes (Dunnington et al., 1989; Martin et al., 1989; Lillie et al., 2017). MHCY was not considered in these earlier studies. In the second population, Wageningen (**WU**) HA and LA lines, chickens were similarly divergently selected over multiple generations for antibody responses to SRBC (Pinard et al., 1993; Parmentier et al., 2004). While these lines are no longer available, archived tissues samples provided the means to retrospectively MHCY genotype HA, LA, and a companion control line. Here, MHCY genotype/haplotype frequencies are defined and analyzed for association with antibody titers in these lines.

MATERIALS AND METHODS

Chickens

The VT HAS and LAS lines were selected at each generation for high or low antibody response to SRBC (Zhao et al., 2012), and all procedures were carried out in accordance with the guidelines established by Virginia Tech Institutional Animal Care and Use Committee. HAS and LAS originated from the same founder population of Cornell Randombred White Leghorn chickens. Each line consists of approximately 120 chickens at each generation. Selection at each generation is based on the responses to single immunizations made between 41 and 51 d of age. Immunizations are made by injecting 0.1 mL of a 0.25% SRBC solution into the brachial vein. Antibody titers are determined 5 d later in 96-well microtiter plate hemagglutination assays (Lillie et al., 2017). All antibody titers are expressed using the exponent of $\log_2$ of the reciprocal of the last dilution in which agglutination was observed.

Initially for the first 9 generations, the VT lines were maintained by selection of 7 males as sires for the next generation. Each male was mated by artificial insemination to 4 females also selected for antibody response to produce the next pedigreed generation. Beginning at the 10th generation, the populations were reproduced using 8 males and 32 females (4/sire). To reduce inbreeding

associated with common ancestry, restricted truncation was used to insure more even representation of families from one generation to the next. Mating full sibs and half-sibs was avoided.

The VT HAR and LAR, serving as controls are sublines established from HAS and LAS lines, respectively, at generation 24. From then on, selection for antibody response was curtailed with the HAR and LAR chickens maintained as closed populations. Within each of these lines, 20 randomly selected females were artificially inseminated with semen pooled from 10 randomly selected males to produce the next generation.

The HA and LA chicken lines at WU were similarly selected for high or low antibody response to SRBC. They originated from the ISA Warren medium heavy brown layer population (Pinard et al., 1993; Parmentier et al., 2004). HA and LA sires and dams were selected based on antibody titers in hemagglutination assays made using plasma collected 5 d after subcutaneous immunization with 1 mL of 25% SRBC. A control line (C) was maintained by random breeding to represent the genetic pool in the founder population. At each generation, 200 chickens were grown for each line. The HA and LA lines were reproduced by the mating of 25 males and 50 females at each generation. Forty males and 80 females were used to reproduce the control line with care taken in the selection of sires and dams to avoid inbreeding. The lines have been discontinued. Frozen tissue samples were available from generations S31 and S32.

DNA Isolation

DNA samples for individuals at generations S44 and S45 in the VT HAS (n = 177) and LAS (n = 187) lines were isolated at VT using Gentra Puregene Cell Kits (Qiagen, Valencia, CA). In the same manner, DNA samples were isolated for individuals in the VT HAR (n = 54) and LAR (n = 56) lines at generation 45 (after 21 generations of relaxed selection in lines initially selected for 24 generations). Frozen tissue samples from generations S31 and S32 of the WU lines were the source of DNA from the HA (n = 68), LA (n = 62), and C (n = 32) lines. These samples were collected and stored frozen in 2011. DNA from these samples was isolated at COH using a DNeasy Blood & Tissue kit (Qiagen).

Identifying MHCY Genotypes and Haplotypes

MHCY genotypes were determined at COH using mSTR typing (Zhang et al., 2020). The STR is located immediately upstream of the MHCY class I loci. The class I loci and their accompanying STR sequences are distributed across the MHCY gene region. They provide information from across the length of haplotypes, an essential element for typing MHCY haplotypes, which can vary widely in size. For this study, we used 2 fluorescently tagged 5'-primers (FAM-897 and YY-916) paired with an unlabeled 3'-primer (899). PCR products from these primers provided chromatograms that allowed the MHCY haplotypes segregating in the fully pedigreed HAS and LAS families to be distinguished. The FAM-897 (identified in blue) and YY-916 (identified in green) labeled PCR products resolved well when co-electrophoresed in a single capillary (Figure 1). Chromatograms were generated in an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and were analyzed with Peak Scanner 2.0. The chromatograms are stable and reproducible from the same sample. The patterns in chromatograms are defined by fragment sizes and peak heights. Family data used to define MHCY haplotypes were from 2 generations of the VT lines, and a few additional special matings needed to confirm haplotype patterns. Haplotypes could then be assigned for all individuals in the HAS and LAS lines. Two additional haplotypes (f and g) in the HAR and LAR lines were inferred from additional patterns present in the chromatograms for these lines. For lack of family data, MHCY data for the WU lines are described only as genotypes.

Statistical Analysis

Differences observed in MHCY genotype and haplotype frequencies between high and low antibody lines in the VT and the WU lines were evaluated using the chi-square test. For genotypes, the chi-square test was applied only to categories containing 10 or more values. Hence, tests were not done for the HAS and LAS categories with fewer than 10 values and for HAR and LAR samples because of small sample size. The significance of the variation of antibody titer among different MHCY genotypes in HAS and LAS lines were evaluated by analysis of variance (**ANOVA**). Mean antibody titers for 2 different MHCY genotypes in the HAS and LAS lines were compared for significant differences using Tukey's test.

Datasets

The study datasets included: 1) Lines selected at Virginia Tech (HAS, LAS, HAR, and LAR) as well as the SRBC antibody titers for individual chickens and 2) lines selected at WU (HA, LA, and C). The model assumed no difference in MHCY genotypes among the lines.

RESULTS

MHCY in the VT HAS and LAS Lines

Initial Tests To begin this project, we mSTR-typed a small sample set of the VT HAS and LAS chickens to explore the potential of using the VT antibody selected lines to evaluate the MHCY haplotype association with immune responses. This exploratory test provided

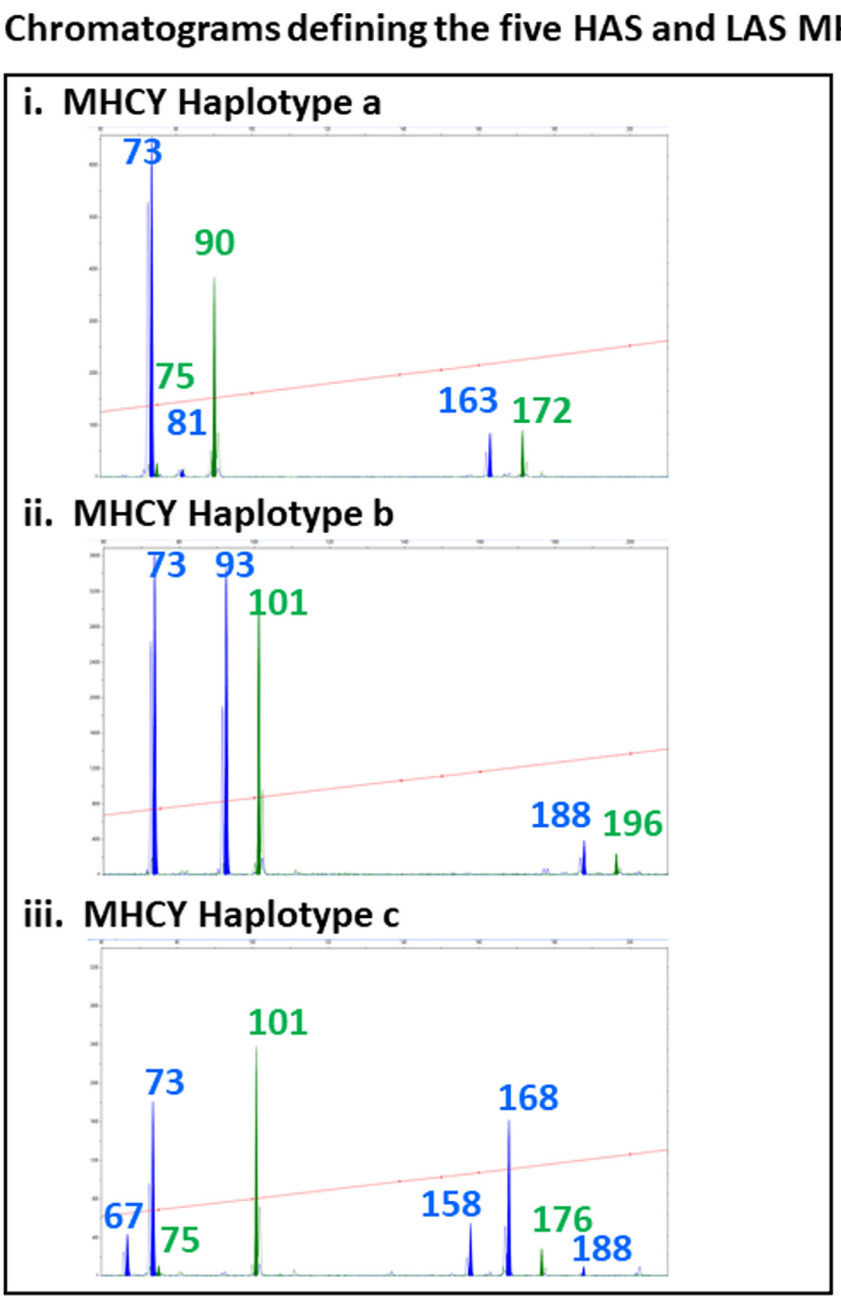
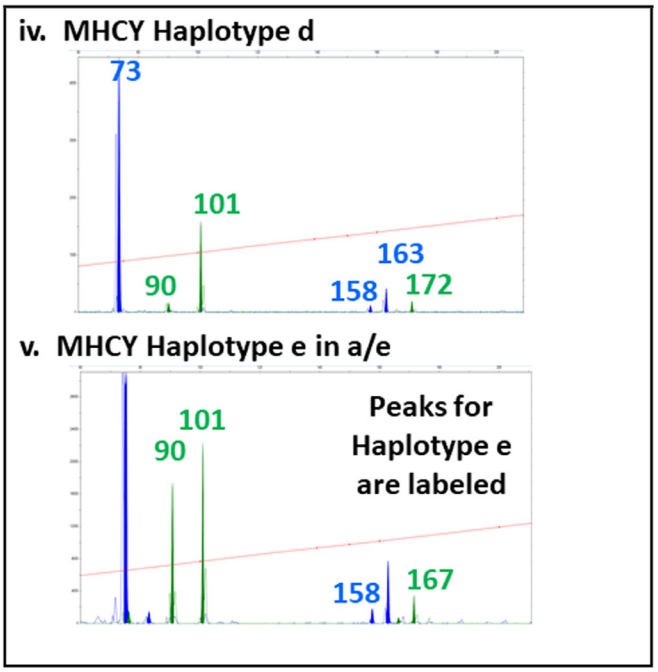


Figure 1. Chromatogram patterns from mSTR typing that define the five MHCY haplotypes segregating in HAS and LAS lines. The presence of five haplotypes were revealed in the analysis of 76 fully pedigreed families. (A) The chromatograms for each haplotype (A−E) contain peaks from PCR products of several lengths that reflect the presence of STR regions of different sizes in each haplotype. The peaks define the five haplotypes. Peaks from PCR amplifications with Primer 897 are in blue. Peaks from PCR amplification with Primer 916 are in green. (B) The numerical values for peaks from the PCR amplifications with primers 897 and 916 provide the tabular patterns that define the five haplotypes.

results suggesting MHCY genotype distribution differences between HAS and LAS lines (data not shown). To follow up on this observation, we defined the mSTR patterns for individual MHCY haplotypes by typing within the pedigreed families in the HAS and LAS lines at generations 44 and 45 plus a few additional special matings to confirm haplotype identification (Table S1, 76 families overall). Chromatograms revealed the presence of 5 patterns for MHCY haplotypes in the HAS and LAS lines (Figure 1).

MHCY Genotypes in the VT HAS and LAS Analysis of the 5 haplotypes revealed a striking difference in their distribution between HAS and LAS chickens at generations 44 and 45 (Figure 2). Haplotypes *a* and *e* are present only in HAS chickens, with the former being the most abundant haplotype. Haplotype *d* is present only in LAS. The haplotypes *b* and *c* were found in both lines, although far more frequent in the LAS line. Overall, the unequal MHCY haplotype distribution in HAS and LAS is highly significant ($P < 0.0001$).



B. Tabulated peak data for the five MHCY haplotypes in HAS and LAS

MHCY haplotype	5' Primer 897 Peak Sizes (bp)								5' Primer 916 Peak Sizes (bp)							WB Number*
	67	73	81	93	158	163	168	188	75	90	101	167	172	176	196	
	Peak Values															
a		18095	256			2005			387	7062			1315			3010
b		20700		17542				2132			11847				982	3286
c	5108	21879			6483		19718	1269	661		15298			1878		5524
d		27128			867	3075				1155	9191		1272			3269
e**		?			1355					6613***	7963	272				5328

*Wing band numbers for individuals scored. All are homozygous except for No. 5328, which is "**a/e**".
** Inferred from families in which "**e**" is segregating. Cannot define 73 bp peak in "**e**" with available data.
***Peak shared with "**a**".

Figure 1 Continued.

Among the genotype scores for all birds in the HAS and LAS lines at generations 44 and 45 (Table S2, Group 1 [177 HAS] and Group 2 [187 LAS]), we found 12 genotypes, all with significantly different frequencies between the lines (Figure 3A). Six genotypes present in the HAS line, *a/a, a/b, a/c, a/e, b/e*, and *c/e*, are not present in the LAS line. Conversely, 3 of the 6 genotypes, *b/d, c/d*, and *d/d*, in LAS line are not found in HAS. Only *c/c, b/c*, and *b/b* genotypes are present in both lines. All 3 shared genotypes were more frequent in the LAS line. Even for *b/c*, the genotype most nearly equally shared, the frequency differed significantly ($P < 0.026$) between lines.

MHCY Genotypes in the VT HAR and LAR Lines To further evaluate the differences observed between the HAS and LAS lines, we MHCY genotyped the VT HAR and LAR lines. Selection in these lines for antibody response was relaxed 21 generations prior, after initial selection for 24 generations for high or low antibody responses (Table S2, Group 3 [54 HAR] and Group 4 [56 LAR]). In all instances but one, the frequencies of the MHCY genotypes in the HAR and LAR lines are significantly different ($P < 0.002$) from their counterparts in the HAS and LAS lines (Figure 3B). Among the genotypes compared, the only genotype not significantly different was *d/d*, found in both LAS and LAR. The HAR and LAR lines have 4 additional genotypes (*b/f, d/f, f/f, f/g, g/g*) not present in HAS or LAS and lack haplotype *c*, providing additional evidence supporting divergence of the lines over time.

MHCY Genotypes and Antibody Titers in VT HAS and LAS Lines Antibody titers to SRBC for each chicken at each generation provided another means by which to evaluate the relationship between MHCY and antibody responses to immunization. Tabulating MHCY genotype versus antibody titer revealed, as expected, differences in titer end point between HAS and LAS lines (Table 1). Within the HAS line, antibody titer end points were quite similar among the MHCY genotypes. Only the highest and lowest titer end points were significantly different. Among the MHCY genotypes in the LAS line, there were no significant differences in antibody titer end points.

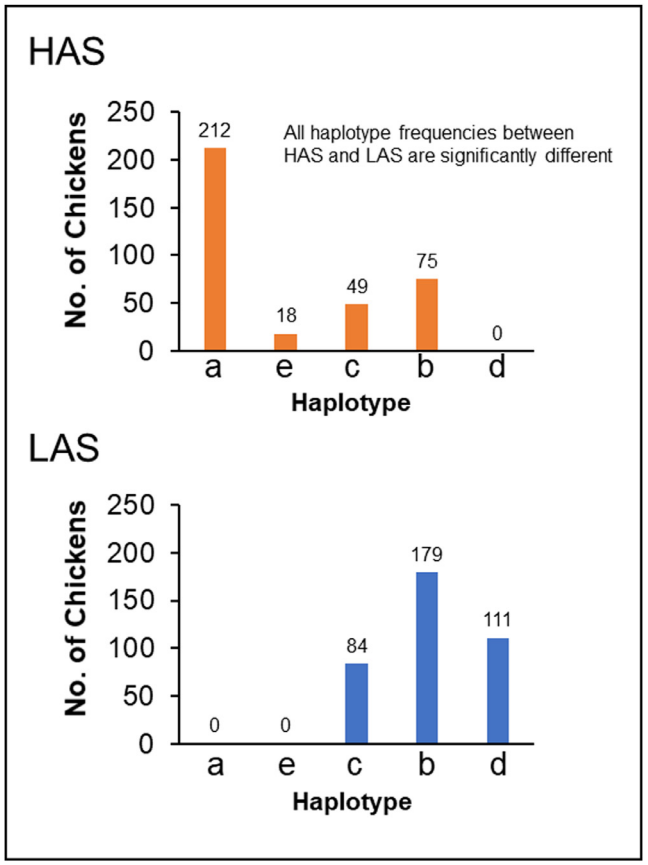


Figure 2. Distribution of the five MHCY haplotypes in the VT chicken lines selected for high (HAS) or low (LAS) antibody response.

MHCY in the WU Lines

An experimental population at Wageningen University selected for high or low antibody responses to SRBC provided a second opportunity to examine the relationship between selection for antibody responses and MHCY genotypes. The experimental design of the WU experiment is similar to that conducted at VT (differences in experimental procedures are as noted in Materials and Methods). Selection for high or low antibody titer led to 2 lines with significant differences in antibody titer. Here too, differences in the distribution in MHCY genotypes are apparent in the 2 lines (Figure 4 with typing data for individual chickens in Table S3). MHCY genotype WU01 was more frequent within the HA line than in the LA line ($P < 0.0001$). Another MHCY genotype WU17 was more frequent within the LA line than in the HA line ($P < 0.00008$). A third MHCY genotype, WU09, was especially common in both the HA and LA lines compared to the control line. The MHCY genotype distribution in the smaller sample set for nonselected random-bred control line shows no similarity to the HA or LA distributions.

DISCUSSION

The data presented here support the possibility that MHCY haplotypes contribute to differences in antibody titers observed in the VT and WU antibody response lines. These positive associations between antibody production and MHCY genotype support further investigation into a role for MHCY in immunity. MHCY is clearly not simply a duplicate of the classic MHCB region in chickens. The 2 regions differ in gene content and organization. The MHCY class I molecules are quite different even though, like classical MHC class I, they are polymorphic and polygenic. Although polymorphic, it is most reasonable to consider MHCY class I molecules as new members of the group of MHC-like molecules emerging as important in presentation of diverse antigens to specialized T cells (Ogg et al., 2019). Monomorphic MR1 molecule that presents microbial riboflavin metabolites to mucosal-associated invariant T (**MAIT**) cells is another molecule in this class (Kjer-Nielsen et al., 2012). Several types of CD1 molecules encoded by monomorphic loci that present a variety of lipids to T cells bearing diverse T cell receptors are included as well.

The features of MHCY class I molecules are consistent with them being similar to MR1 and CD1 but going beyond because of their polymorphism. If they have a role in early signaling, their polymorphism would confer a degree of specificity to innate immune responses not previously observed. The signaling might occur by MHCY class I molecules being loaded with a specific ligand. Then the loaded molecules might be displayed more prominently (with greater stability or with increased concentration) on the cell surface so that specialized immune cells with cognate receptors recognize the signal and relay the message to other immune cells responsible for memory-based immune responses. The idea of specificity provided by MHCY polymorphism in early recognition events is intriguing. Whether the outcome is activation of an immune response eventually resulting in antibodies, other cellular immune responses

Table 1. Mean antibody titer among MHCY genotypes in chickens selected for high (HAS) or low (LAS) antibody response to sheep red blood cells (SRBC).

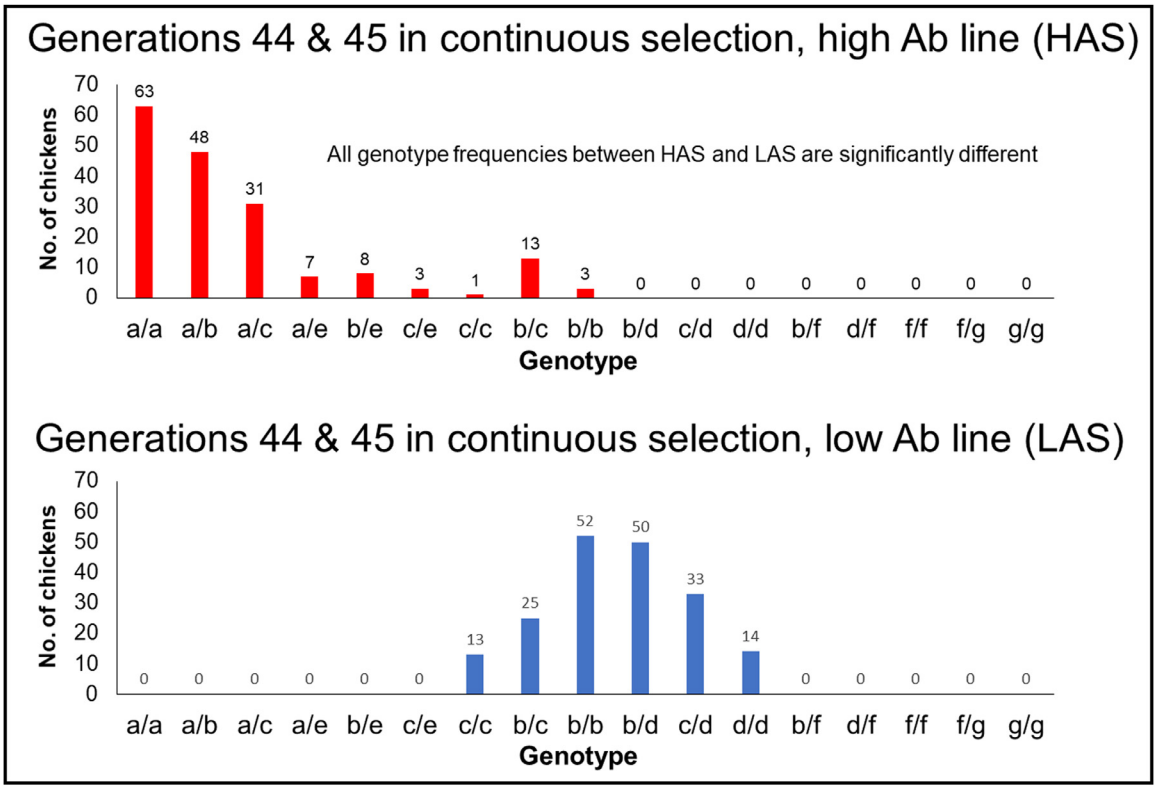
MHCY genotype	Line	Count	Mean ± SD
c/e	HAS	3	24.67 ± 6.66^{a}
b/b in HAS	HAS	3	18.67 ± 6.43^{ab}
c/c in HAS[1*]	HAS	1	18.00
a/a	HAS	63	16.97 ± 5.81^{ab}
a/b	HAS	48	16.00 ± 5.04^{ab}
a/e	HAS	7	15.71 ± 2.63^{ab}
a/c	HAS	30	15.60 ± 5.36^{ab}
b/c in HAS	HAS	13	15.00 ± 6.40^{ab}
b/e	HAS	8	13.13 ± 6.22^{b}
c/c in LAS	LAS	13	3.62 ± 1.98^{c}
b/d	LAS	50	2.74 ± 1.82^{c}
d/d	LAS	14	2.71 ± 1.64^{c}
b/c in LAS	LAS	25	2.60 ± 2.08^{c}
c/d	LAS	33	2.58 ± 1.56^{c}
b/b in LAS	LAS	52	2.52 ± 1.77^{c}
ANOVA F critical value=1.75***			

Antibody titers were measured 5 d after intravenous injection of 0.1 mL 0.25% SRBC.

[abc]Means having no common letter are significantly different ($P < 0.025$).

[1*]Single sample not included in statistical analysis.

***P-value< 0.001.



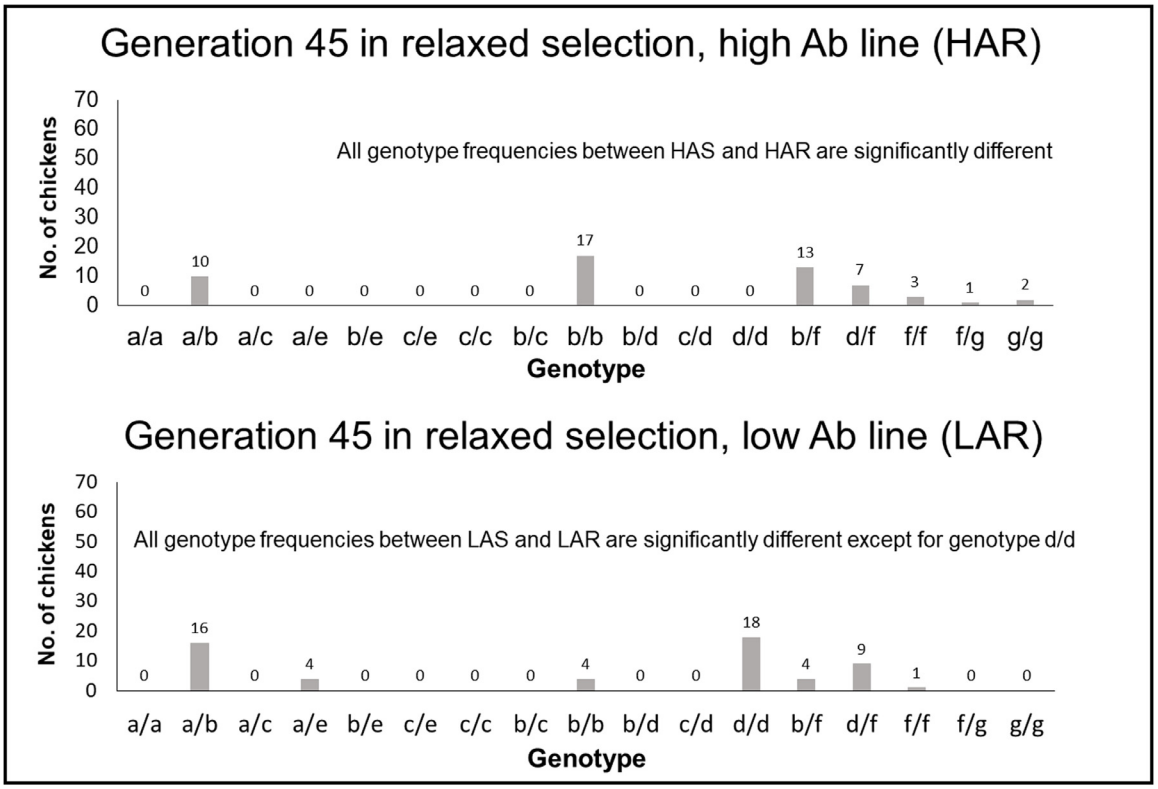


Figure 3. Comparison of the distribution of genotypes in the VT chicken lines selected for high (HAS) or low (LAS) antibody response with the HAR and LAR lines in which selection was relaxed.

or inhibition of an immune response could be dependent on the specificity of the signaling MHCY molecules. MHCY molecules with antigen might send activation signals to specialized T cells as occurs with MR1 (Godfrey et al., 2019), or they could provide inhibitory signals, similar to the interactions between MHC class I and receptors on natural killer cells (Saunders et al., 2015).

More experiments are necessary to test the association between MHCY and antibody production. With

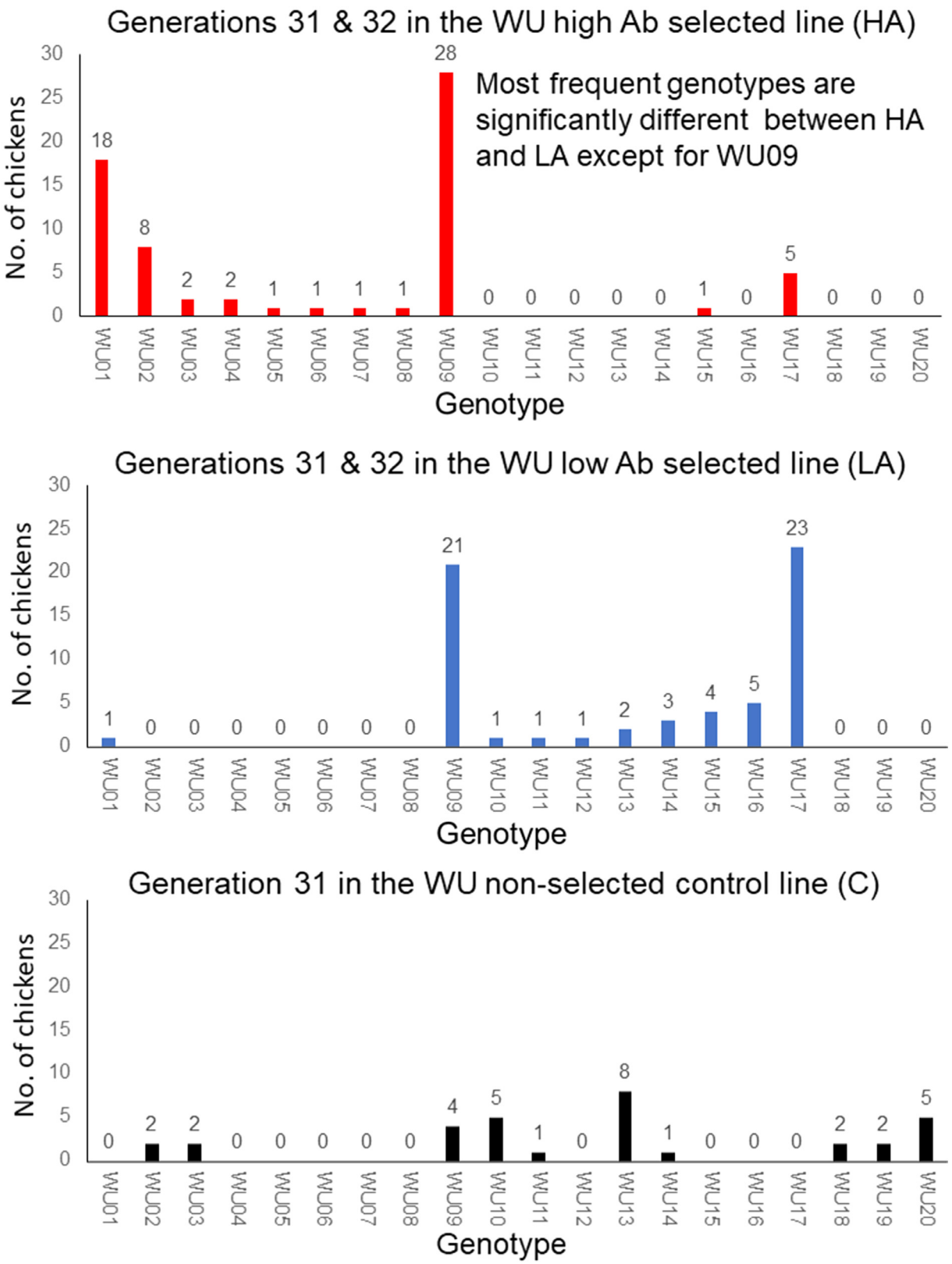


Figure 4. Distribution of MHCY genotypes in the WU high and low antibody selected lines (HA and LA) and unselected control line originating from the same genetic stock.

individual haplotypes identified as candidates guiding high and low responses to SRBC, it may be possible to further test the role of MHCY by making crosses between chickens with candidate MHCY haplotypes, such as within the VT HAS and LAS lines, controlling for MHCB and other candidate loci (Lillie et al., 2017), and testing progeny for responses to immunization with SRBC. If the association of antibody responses against SRBC with individual haplotypes is upheld in tests with these intercrosses, then subsequent studies can focus on MHCY in guiding immune responses to other antigens, including microbes that affect chicken health. Previous

work has shown that the VT and WU high and low antibody lines differ in responses to common poultry pathogens (Gross et al., 1980; Berghof et al., 2019).

Our experiments provide evidence for a link between MHCY haplotype and immune responses to the experimental antigen, sheep red blood cells. Associations observed in 2 independent experiments with genetically dissimilar populations increase the likelihood that the observations linking MHCY haplotypes to immune responses is real. If MHCY class I molecules do telegraph the presence of microbes, MHCY genetic differences among individuals could be of interest in the selection of genetically resistant strains of chickens.

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DISCLOSURES

The authors have no conflicts of interest to report.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101621.

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