

## Review

## Evolutionary pressures rendered by animal husbandry practices for avian influenza viruses to adapt to humans

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## SUMMARY

**Commercial poultry operations produce and crowd billions of birds every year, which is a source of inexpensive animal protein. Commercial poultry is intensely bred for desirable production traits, and currently presents very low variability at the major histocompatibility complex. This situation dampens the advantages conferred by the MHC's high genetic variability, and crowding generates immunosuppressive stress. We address the proteins of influenza A viruses directly and indirectly involved in host specificities. We discuss how mutants with increased virulence and/or altered host specificity may arise if few class I alleles are the sole selective pressure on avian viruses circulating in immunocompromised poultry. This hypothesis is testable with peptidomics of MHC ligands. Breeding strategies for commercial poultry can easily and inexpensively include high variability of MHC as a trait of interest, to help save billions of dollars as a disease burden caused by influenza and decrease the risk of selecting highly virulent strains.**

## INTRODUCTION – THE POULTRY INDUSTRY AND AVIAN INFLUENZA VIRUSES

Consumption of animal protein was crucial for the evolution of humans (Finch, 2010; Williams and Dunbar, 2014), but until recently, this food was scarce for many consumers. Since the beginning of the 1900s, improvements in productivity resulting from breeding and management practices of livestock have increased food security, reduced costs, and increased consumption (BBC, 2019; Perren, 1978). As a consequence of improved nutrition and health, decreases in morbidity and mortality of humans owing to infectious diseases followed this transition (Armstrong et al., 1999; Williams and Hill, 2020). The first technological innovation in meat production, the domestication of animals during the Neolithic, fermented denser populations of humans and animals, but also created opportunities for animal viruses to acquire humans as hosts; trades spurred by these changes helped spread the new diseases across human populations (Morand et al., 2014; Wolfe et al., 2007).

As we will argue herein, contemporary farming practices may also generate negative trade-offs: they create higher stress and inherently decrease genetic variability, including at the naturally highly polymorphic major histocompatibility complex (MHC) genes, impairing the immunity of production animals and concomitantly exerting undesirable selective pressures upon pathogens. In this review, we focus on poultry and avian influenza A viruses (IAV). We present the hypothesis that high stress and low genetic variability in commercial breeds of poultry has the potential to generate immune pressures that select variants of avian IAV with altered host ranges and levels of virulence, causing the unpredictable influenza pandemics. The proposed model for this process is depicted in Figure 1. We will present data indicating (i) that genetic variability at the MHC is lower in the highly productive commercial breeds of poultry relative to indigenous chickens, (ii) low genetic variability at the class I MHC can undesirably select pathogens, and (iii) it is possible for cytotoxic T cells to select mutants of IAV proteins that affect host specificity. We also present some research strategies to address this hypothesis and alternatives to mitigate the effects of current production practices.

Livestock genetic improvement programs generate highly productive breeds and genetic lines, selected for performing in production-related traits, such as growth rates, resistance to disease, high fertility and

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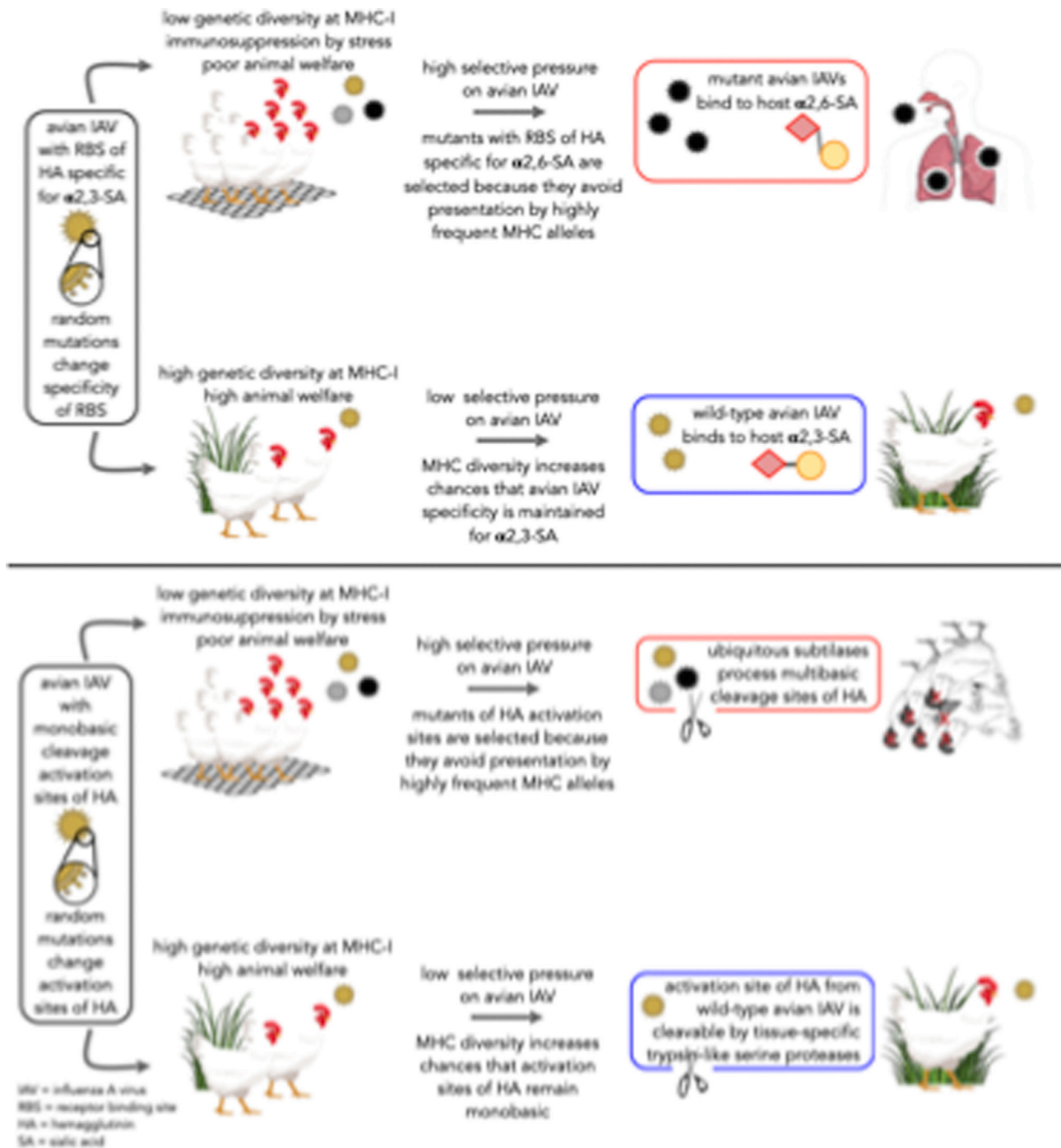
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**Figure 1. Emergence of strains of avian influenza A viruses with new host specificities and increased virulence: possible mechanisms for selection by animal husbandry practices that reduce host genetic diversity and compromise immunity**

Top panel: mutations may modify specificity of binding sites of HA or NA from  $\alpha 2,3$ -SA to  $\alpha 2,6$ -SA directly or indirectly through changes in epistasis between HA and NA, or in NP, M proteins, and polymerases, which affect outcomes of genetic events in IAV. Bottom panel: Mutations affecting cleavability of HA by different proteases affect tissue tropism and thus transmissibility, as well as virulence. The figure was created with MindGraph.

fecundity, and feed conversion rates. As a result, the time to produce, for example, a broiler (meat) chicken decreased by almost 400% between 1950 and 2005 (Zuidhof et al., 2014) accompanied by a 300% increase in the growth rate (Knowles et al., 2008). These selected production traits have had

positive impacts on food security because of huge improvements in productivity and overall production, which are essential to consistently supply worldwide markets with low-cost animal protein for human consumption.

Maintaining high variability of genes encoding the antigen peptide-presenting proteins of MHC within commercial poultry breeds is not considered a production trait; therefore, no scientific publications by academia or industry are available. Genetic association studies with experimental or commercial poultry populations, using sparse molecular marker maps or sole candidate-gene markers, suggest that MHC alleles can be associated with certain production traits of poultry, in addition to the resistance to disease caused by infectious organisms, such as body and egg weight and egg laying (e.g., [Kim et al., 1989](#); [Lakshmanan et al., 1997](#); [Lundén et al., 1993](#)). However, these findings have subsequently had little or no practical application in commercial breeding programs that have gained in productivity using trait measurements and pedigree information. However, MHC B congenic chicken lines have been used to study genetic mechanisms of resistance to different infectious diseases of chickens and MHC B haplotypes, which have been effectively associated with genetic resistance or susceptibility to several diseases of interest to the industry, and indeed are selected for this purpose in commercial breeding programs (reviewed by [Silva and Galardo, 2020](#)).

Poultry production systems raise billions of animals each year. According to the Food and Agriculture Organization (FAO) of the United Nations, the total production of poultry meat in 2020 is estimated to have been 134 Mt, and was underpinned by the rise in demand in China owing to an outbreak of African Swine fever in that country. Poultry meat will continue to be the primary driver of growth in worldwide meat production. Importantly, poultry represents an astounding 70% of all live birds ([Food and Agriculture Organization, 2021](#)). Importantly, live poultry markets have been shown to be primary drivers of evolution of avian IAVs, and the consequent outbreaks of avian IAV in humans ([Gao, 2014](#); [Su et al., 2015](#); [World Health Organization, 2016a, 2016b](#)).

In an evolutionary context, current poultry breeding practices have been in place for a short period of time, but they have promoted great genetic uniformity within a huge population of a single species ([Food and Agriculture Organization, 2021](#)). This fact, added to the speed with which improvements have been achieved and the sheer numbers of commercial poultry alive at any given moment (according to [Gridded Livestock of the World, GLW3 \[2020\]](#), 29 billion chickens lived in 2019), mostly crowded in production facilities, may present an unprecedented selective pressure on pathogens, including avian IAV. It is noteworthy that a typical industrial poultry production facility may house, in partially or completely enclosed buildings, 20,000–26,000 animals from a single breed, at densities exceeding 14 birds/m<sup>2</sup> ([The Poultry Site, 2022](#)).

Only a few decades ago, China's poultry sector relied on millions of smallholder farms and meat and eggs were a luxury good. In the 1980s, poultry production intensified in China and now comprises large-scale intensive industrialized operations that are spatially concentrated; however, the trading practice of live poultry markets has persisted. Southern China is an epicenter of new strains of avian IAV owing to its large poultry industry, proximity to breeding sites of migratory water fowl, and also trading style in live poultry markets ([Bingsheng and Yijun, 2007](#)). Epidemiological investigations of low- and high-pathogenic influenza viruses (LPAIVs and HPAIVs, respectively) confirm that live poultry markets provide opportunities for genetic reassortment of strains and also for exposure of humans (e.g., [Bi et al., 2016](#)). Many reassortant viruses present mutations that result in adaptations to infect mammals (e.g., [Yang et al., 2017](#)). Human workers in the industrialized operations, and not only those in China, can also be exposed to, and have been shown to be infected with, avian IAVs ([Philippon et al., 2020](#)) and even transmit infections with avian IAVs to human contacts ([Brown, 2004](#); [Koopmans et al., 2004](#)).

### THE SELECTIVE ADVANTAGES OF HIGH GENETIC VARIABILITY IN IMMUNE RESPONSE GENES

“Red Queen” dynamics govern how pathogens and hosts co-evolve: pathogens establish molecular interactions to specialize in confronting immune responses of specific hosts ([Phillips, 2002](#)). Genes encoding the antigenic peptide-presenting proteins of the MHC present the largest diversity of all vertebrate gene families ([Radwan et al., 2020](#)). The MHC is naturally highly polymorphic in nature because this situation confers an adaptive advantage to species ([Meyer and Thomson, 2001](#)). Most of the non-synonymous substitutions of amino acids in MHC proteins occur within their antigen peptide-anchoring grooves, suggesting that they

confer Darwinian advantages; heterozygosity within individuals and polymorphisms within a population hamper pathogens' escape from immune recognition because a large array of their epitopes will be presented to collective immune systems of populations.

Mathematical modeling has the advantages of high levels of polymorphisms in the MHC: in events imposing high selective pressures, rare alleles persist in host populations if pathogens manage to evade presentation by the most common alleles (Borghans et al., 2004). Observations in natural populations of several species of animals confirm these hypotheses: populations of naive fish bearing MHC alleles that are novel for a specific pathogen will present reduced infection intensities when infected with this pathogen (Phillips et al., 2018); in wild hare (Awadi et al., 2018) and cheetah (Castro-Prieto et al., 2012) populations, diversity within MHC decreases with decreasing selective pressure by pathogens. Upon their expansion from the Balkans toward central Europe, golden jackals presented a reduced variation in DLA-DQA1 exon 2. This decrease in variation was also affected by positive selection to adapt to different ambient temperatures, which also affects the types of pathogens that will challenge these animals (Stefanović et al., 2021). Very low genetic diversity and a unique MHC (Drb) haplotype in a population of Alpine ibex seem to be the cause of these animals' susceptibility to brucellosis and their role as a reservoir for virulent outbreaks of this disease (Quémeré et al., 2020).

So far, few studies have addressed the reverse aspect of this issue, i.e., the selective pressure mediated by highly frequent MHC alleles upon pathogens for human and animal hosts. Pathogens are expected to adapt by evading presentation by the most common MHC types (Bodmer, 1972). Indeed, Epstein-Barr virus circulating in human populations where the HLA A11 allele is very frequent has mutated and selectively lost anchor residues that bind to this peptide-presenting groove (De Campos-Lima et al., 1993, 1994). Likewise, the simian immunodeficiency viral (SIV) genome diversifies according to MHC-I haplotypes present in macaque populations (Hau et al., 2019). Thus, pathogens can also escape presentation by MHC molecules when certain alleles are highly frequent in a population.

Furthermore, and to the best of our efforts in reviewing the literature, research that seeks to establish if high frequency of an MHC allele can change *host specificities* of pathogens has never been undertaken. If this hypothesis is correct, the consequences can be serious and, for this reason, it is worth being tested. As argued by Wahl et al. (2009), variability at host class I MHC is a foil for virus variability so it must be preserved in a species. Indirect evidence for this is provided by the recuperation of diversity at the MHC and fitness against pathogens in populations of wildlife that underwent bottlenecks (see e.g., Hawley and Fleischer, 2012; Oliver and Piertney, 2012). Until our hypothesis is tested, one cannot state that a host switch in IAV owing to genetic uniformity of MHC genes in poultry is impossible because, to the best of our knowledge, the scientific community has never specifically studied this event, not even with mathematical modeling.

## SELECTIVE BREEDING, GENETIC IMPROVEMENT, AND GENETIC VARIABILITY IN FARM ANIMALS

Klein (1987) observed that "any theory of MHC polymorphism must explain five fundamental observations." One is that "the degree of MHC polymorphism is not influenced in any striking way by the ecology of the species." However, low diversity in antigen-binding sites of MHC in certain species of wildlife points to demographic processes (Castro-Prieto et al., 2011). The composition of MHC alleles in old and new urban and rural populations of wild waterbirds depends on the level of anthropogenic disturbance (Pikus et al., 2021). The diversity of MHC in pigmy hogs that reproduce in captivity is reduced after only eight generations (Purohit et al., 2021).

Current practices for producing poultry and swine also suggest that Klein's observations should be reassessed because animal husbandry practices do provide an ecology for commercial poultry such that it seems to affect the levels of polymorphisms at the MHC. In chickens, MHC haplotypes include the classical polymorphic class I and class II genes and the polymorphic peptide-loading genes, all of which are in strong linkage disequilibrium (Kaufman, 2018). Regarding MHC polymorphisms in chickens, Ewald et al. (2007) typed BF2 (the class I loci that corresponds to HLA-A and -B and restricts the antigen-specific cytotoxic T cell [CTL] immune response) alleles in 100 sires each of three purebred commercial broiler chicken lines: 11 BF2 alleles were identified. No allele was common to all three lines; only three BF2 alleles were shared by two of the three lines and each line presented three, five, or six alleles, respectively. In another example, Izadi and colleagues (2011) observed that more alleles were found in noncommercial populations of

chickens than in commercial breeds. The 66 genotypes accounted for in the eight chicken populations that those authors studied were generated by only 22 alleles. Chazara and colleagues (2013) also observed that the degree of polymorphism at the chicken MHC differs between commercial and native populations of chickens breeding under natural circumstances, being much lower in the former birds. A study of 21 chicken populations in China examined the diversity of SNPs in the 92 kb MHC B-F/B-L region containing 18 genes (Yuan et al., 2021). It confirms that highly selected genetic lines have much lower diversity and indigenous Chinese domestic chicken breeds have the highest diversity in this genome region. In all, these data indicate that variability at the BF2 locus in pure commercial lines of chickens is low, but, to the best of our knowledge, there are no published studies formally addressing the issue of frequencies of BF2 alleles in these lines nor in populations housed in an industrial facility.

Intense genetic selection, powered by assisted reproduction technologies, has resulted in significant reductions in effective population sizes ( $N_e$ ) and genetic variability of livestock.  $N_e$  as low as 48 (Zanella et al., 2016) and 32 (Márquez et al., 2010) are reported for commercial swine and chicken populations, respectively, owing to line-breeding practices. To give readers unfamiliar with population genetics and animal breeding an idea of this dimension (see Wright, 1938), the estimated  $N_e$  of Japanese inhabitants in Tokyo is 3,100 individuals (Tenesa et al., 2007.). The  $N_e$  for the critically endangered swift parrots of Australia has been estimated to be between 48 and 140, depending on the method employed (Olah et al., 2020). The size of linkage disequilibrium blocks and the number of haplotypes at the MHC found in populations of a species is an indicator of their  $N_e$  and, generally, the smaller the LD blocks and the greater the numbers of MHC haplotypes, the greater the corresponding  $N_e$  is. Of relevance for this review, a study of jungle fowl from four wild populations (Nguyen-Phuc et al., 2016) presented strikingly higher numbers of haplotypes (in between 100 and 78% of the sampled animals, which presented 310 unique haplotypes among them) than jungle fowl bred in captivity (43% presenting five unique haplotypes; Athrey et al., 2018) and even more so than commercial lines of chickens (between 1.9 and 5.4%; Nguyen-Phuc et al., 2016). Whereas undesired reductions in MHC variability may not directly impact productivity, long-term consequences for animal and human health may be significant (Alexander, 2007).

Genetic selection and improvement strategies for growth traits in poultry genetic lines are usually performed in environments with low levels of microbes and Specific Pathogen Free-conditions (bio-secure conditions). Thus, to improve disease resistance, breeding programs couple this with sib-testing, where sibling animals are exposed to more challenging environments, with microbe loads similar to normal production conditions, to estimate the genetic values and select the superior, but unexposed full and half-sibs that remained in the breeding nucleus. These are common processes widely used in breeding and are employed in this instance to improve resistance to certain infectious diseases of commercial genetic lines and breeds (Chu et al., 2018). However, although animal health is intrinsically correlated with productivity and included in selection objectives in all breeding programs, to the best of our efforts, we did not find reports citing direct selection for *high* variability of immune system components in poultry or swine. Importantly, as mentioned above, commercial chicken lines have been shown to have significantly fewer MHC polymorphisms and MHC haplotypes than native populations and traditional breeds of chickens (Chazara et al., 2013; Izadi et al., 2011; Yuan et al., 2021). The fact that entire flocks in commercial operations are decimated during avian flu outbreaks could be a reflection of low genetic variability in the MHC and other immune response genes (Muir et al., 2008).

## IMPACT OF FARMING MANAGEMENT PRACTICES ON IMMUNITY

Synergizing with breeding practices are the intensive management conditions (reviewed by Karcher and Mench, 2018) that also seek to reduce production costs in order to satisfy consumers. These practices induce stress in the animals. High stocking densities, long periods of transportation, toxicological stress (such as high concentration in the air of ammonia from excreta and high concentration of antibiotics in poultry feed), and pre-slaughter handling are the main categories in the poultry industry (Place and Mitloehner, 2014). Chickens under heat-stress had higher levels of plasma corticosterone, produced lower levels of antigen-specific IgM and IgY, and of IgY upon immunization with BSA, presented atrophy of lymphoid organs, and had lower counts of precursor T cells ( $CD4^+CD8^+$ ) in the thymus (Hirakawa et al., 2020); chickens under heat-stress also had higher pathogenic bacteria counts in the gut (Song et al., 2013), increased bacterial translocation to other organs (Hirakawa et al., 2020), increased infiltration of heterophils and lymphocytes in the small intestine, and higher levels of TNF-alpha and IL-6 in the plasma and small intestine (Quinteiro-Filho et al., 2010; Wu et al., 2018). Stress increases corticosterone levels six-fold,

and suppresses glycogenolysis and glycolysis, causing alterations on striated skeletal muscles in chickens (Hazard et al., 2011). These findings support a deficient adaptive immune response. Importantly, obese humans infected with IAV present prolonged viral shedding, possibly owing to compromised immunity to the virus including increased levels of TNF- $\alpha$  and IL-6 (Honice and Schultz-Cherry, 2019). It is not known if compromised immunity increases viral shedding in poultry, but stress decreases the number of CD8<sup>+</sup> cells (Hirakawa et al., 2020). In addition, excess fat deposition and fatty liver hemorrhagic syndrome is a current problem in laying hens (Guo et al., 2021). Increased viral shedding may increase the chances of spreading recombinant viruses. The result of these stress-inducing, “immunosuppressive” (Glaser and Kiecolt-Glaser, 2005) management practices may be that the sole selective pressure on a strain of avian IAV are the collective MHC class I phenotypes. Stress (Truckenmiller et al., 2005) and IAV itself (Koutsakos et al., 2019) reduce surface MHC class I; however, the effect is moderate and immunosuppression does not change the peptide-binding pockets of MHC class I alleles, nor abrogates the presentation of IAV peptides by MHC class I.

Sanitary measures adopted by commercial farms for finishing swine and poultry are unable to avoid the introduction of pathogens owing to varying standards in management practices and ecological circumstances, attested to by the epidemiological characteristics of the 2020–2021 epidemic of avian IAV in Europe (European Food Safety Authority et al., 2021). Moreover, commercial operations raising animals in lower scale, free-range systems, which are even more exposed to pathogens (e.g., introduced by wild fowl), frequently utilize germplasm from commercial poultry lines, to use their increased genetic potential for higher productivity (Leenstra et al., 2016).

## BIOLOGY AND EVOLUTION OF IAHS WITH A FOCUS ON AVIAN IAHS

Understanding how avian IAHS cross species barriers and cause pandemics is an active area of research. The question arises: are there immune selective pressures for the variants of avian IAV that are able to infect humans and, if so, what are they? Herein we argue how low genetic variability of avian MHC, especially at BF2, in commercial poultry could mediate the adaptation of avian IAHS to humans. Solid arguments must address the biology of IAV proteins and how they can suffer selective pressures from host CTL responses: their functions must be understood because features of several viral proteins determine host specificity and transmissibility.

First, mutant avian viruses must be able to replicate at the comparatively lower temperatures of the human upper respiratory tract in order to maintain transmissibility. This characteristic is dependent on viral polymerase, which presents poor proofreading. Second, mutant avian viruses must change the specificity of its receptor-binding sites (RBS), including its receptors for structures of the upper respiratory tract to permit infections in humans and to facilitate human-to-human transmission. Concomitant to this change, the viruses must compensate for the changes in RBS with changes in other parts of their structure in order to maintain the RBS’s stability. The two latter features are dependent on hemagglutinin (HA) and possibly neuraminidase (NA) owing to the epistasis between these two RBS (Kosik and Yewdell, 2019; Thyagarajan and Bloom, 2014). However, if an avian IAV is to generate a productive infection in humans, the catalytic activity of NA obviously must remain and be preserved from mutations. HAs and NAs present inherent greater tolerance for mutations than other proteins of IAHS (Kosik and Yewdell, 2019; Thyagarajan and Bloom, 2014). In the next topic, we will describe the ligand specificities of these two RBS. The nuclear protein (NP) and matrix proteins (M1 and M2) affect the packaging efficiency of the IAV’s segmented genome and, consequently, viral fitness (Brooke et al., 2014). Regarding the non-structural proteins (NSPs) of IAV, besides determining its pathogenicity and modulating its life cycle, they provide evasion of host innate defenses (Hao et al., 2020), which could render innocuous changes in the species-specificity determining proteins HA and NA.

Another aspect that determines host-specificities of IAHS are the host’s proteins of innate immunity that restrict the virus. In the context of our hypothesis and in order to facilitate adaptations of the avian strains to humans, mutations that direct affinities of the viral proteins listed above from avian toward human proteins must also occur. Host importins transport protein molecules from the cell’s cytoplasm to the nucleus and viral PB1-F2 and N40 bind to it (Gabriel et al., 2008). Avian H7N9 IAV, which has caused fatal infections in humans, has already adapted to use human importin- $\alpha$ 7 and leads to high levels of virus replication in human lung cells (Bertram et al., 2017). MxA host proteins participate in intracellular vesicle trafficking and seem to restrict IAV by binding to viral NP and blocking the early steps of the viral life

cycle (reviewed by [Haller and Kochs, 2020](#)). Mutations in NP from avian-like swine IAV that escape restriction by human MxA proteins have been described, representing a further opportunity for host switches in avian IAVs ([Dornfeld et al., 2019](#)). Host retinoic acid-inducible gene (Rig-I) and melanoma differentiation-associated gene 5 (MDA5) are RNA helicases that detect certain structures of viral RNA and activate the innate immune response ([Kato et al., 2006](#)). Since viral RNA will not be presented to CTLs via avian MHC class I, these interactions are probably not involved in adaptations of the avian IAV strains to humans. Interestingly, Rig-I, while present in ducks and other water-fowl, seems to be absent in chickens. This fact may explain the relative resistance of the former species of birds to symptomatic AIV infections, and their role as reservoirs of IAVs and the exquisite susceptibility of chickens to these viruses ([Barber et al., 2010](#)). In order for IAVs to access their hosts' cytoplasm and introduce their genetic information, they exploit the lowering of the pH in the endosomal compartment. This lowering in pH causes the necessary conformational changes of their HA that lead to the fusion of the viral membrane with the host endosomal membrane, with access into the target cell cytoplasm (reviewed by [Caffrey and Lavie, 2021](#)). Obviously, mutations that lead to changes to certain amino acids of HA can affect the characteristics and, consequently, conformational changes of HA that occur in the endosomal compartment. Importantly, different activating pH seem to be involved in adaptation to host species: HA-activating pH values are lower in IAVs adapted to the human host ([Russier et al., 2016](#); [Peacock et al., 2017a](#)). In avian strains, LPAIV present a lower activating pH, whereas H5 HPAIV exhibits a higher pH ([DuBois et al., 2011](#)). Furthermore, different host tissues display different final pHs in their endosomes, which may explain in part the observed tissue tropism as well as host specificity of strains of IAVs ([Daidoji et al., 2015, 2017](#)).

IAVs evolve through two complementary genetic mechanisms that affect virulence, transmissibility, host range, and potential to cause pandemics (reviewed by [Goneau and colleagues, 2018](#)). One mechanism is genetic drift, where mutations accumulate owing to the error-prone polymerase mentioned above and are then negatively (unfit virus) or positively (fitter virus) selected by host immune pressures. Advances in sequencing techniques have facilitated obtaining numerous full genomes, and this approach, when applied to animal-associated IAV, has revealed many human-adaptive mutations accumulating and circulating within animal populations, including chickens. Upon transmission to a human host these mutated viruses rapidly expand ([Munoz et al., 2016](#)). Another genetic mechanism also changes biological characteristics of IAV and relies on its segmented genome and needs at least two strains of avian IAV infecting a host cell, where they can exchange segments and undergo reassortment. Genetic reassortment of IAVs has been the major driver of influenza pandemics since the 1918 pandemic ([Goneau et al., 2018](#); [Tang et al., 2010](#)).

Importantly, [Beerens and colleagues \(2020\)](#) have shown that even when a strain of avian IAV (in this case, HPAIV) represents the minority of viral isolates from infected birds on a farm it is rapidly selected in experimentally infected birds inoculated with a mix of viruses. They also observed that the selected molecular changes involved deletions in the stalk region of NA and mutations in HA and PB1 in the HPAIV, all of which were already present in the minority variants.

Low packaging efficiency creates virions lacking gene segments that are, therefore, non-infectious (or "semi-infectious" according to some authors), but that may be rescued by repackaging. A single host cell can be infected by viruses of different genotypes and this fragmented organization of RNA facilitates rapid mixing of different genomes, generating many types of progeny. Non-infectious particles also reassort in the host ([Brooke et al., 2014](#); [Ince et al., 2013](#)). Thus, IAVs evolve through many strategies, varying from amino acid substitutions to reassortment of their segmented genomes, to rescuing of non-infectious virions by replacing genome segments that they were lacking with new segments that differ in sequence. Transmissibility will also be important to promote further mixing of viral genomes.

### The specificities of IAV hemagglutinins and neuraminidases for host sialic acids

The host range of IAVs is determined by the binding specificities of two viral receptors for sialic acids (SAs) present at the ends of glycans on membrane proteins of host cells. SAs are species-specific ([De Graaf and Fouchier, 2014](#); [Varki and Schauer, 2009](#)). The avian and human ligands for HA and NA are 3'-sialyl-acetyl-lactosamine ( $\alpha$ 2,3-SA) and 6'-sialylacetyl-lactosamine ( $\alpha$ 2,6-SA), respectively; avian  $\alpha$ 2,3-SA also presents a sulphated version that has been shown to present preferential binding by certain avian IAVs ([Peacock et al., 2017b](#)). Lectin binding studies show that  $\alpha$ 2,3-SA may be present in humans depending on the anatomical

location (upper or lower respiratory tract) and age of the subjects (adults or children; [Nicholls et al., 2007](#)). Viral HAs and NAs are the RBSs that discriminate the structures of SAs ([De Graaf and Fouchier, 2014](#); [Xiong et al., 2014](#)); HA and NA promote, respectively, the infection of host cells by binding to SAs and, subsequently, releasing newly formed virions through cleavage of SAs.

Antibodies are the main selective pressure on HA and NA and drive the antigenic drift of IAVs. Antibody-mediated selection of these mutants affects the efficacy of vaccines against viral strains, but does not necessarily change their host range. However, new selectivities of HA and NA for alternate SAs present in a different host species may be one consequence of this pressure. In fact, between 1972 and 2013, three amino acid substitutions arose in the avian IAV H6N1 RBS that resulted in a strain that binds to human SA; the first human case of infection with avian influenza A H6N1 virus was reported in Taiwan in 2013 ([Wang et al., 2015](#)). Over these four decades, the receptor-binding properties of the HAs of the Taiwanese H6 isolates first presented the capacity to bind to avian SA, then obtained the additional capacity to bind to both human and avian SAs and then acquired a binding preference for human SA. A mutagenesis-type study determined that E190V and G228S substitutions in HA are important to acquire the capacity to bind to the human receptor; the P186L substitution could reduce the binding to the avian receptor. In 2014, [Wang et al. \(2014\)](#) isolated 257 H6 strains from live poultry markets in China and tested them for binding specificity; 87 (34%) were able to bind to human SA. Genome sequence analysis indicated that these viruses are actively circulating and reassorting in nature.

Another example of evolution in IAVs affecting the host range involves the H2N2 and H3N2 viruses that have caused pandemics: the HAs of these strains were of avian origin ([De Graaf and Fouchier, 2014](#)). Two mutations in the receptor-binding site of the HAs were sufficient for these avian viruses to switch from being specific for  $\alpha$ 2,3-SA, abundant in avian respiratory and intestinal tracts, to  $\alpha$ 2,6-SA, abundant in human upper respiratory tracts.

Highly pathogenic avian influenza A/H5N1 virus (HPAI A/H5N1) causes devastating outbreaks in poultry and, eventually, human cases. Several RBSs of HPAI H5N1s infecting humans presented variations at positions that determine the species-specificity of RBS. None of the substitutions resulted in human-to-human transmission. Acquisition of human-type receptor specificity requires multiple amino acid mutations; furthermore, variability at key position 226 of the RBS in HA is not tolerated if IAV is to infect chickens, reducing the risk of the HPAI A/H5N1 being acquired naturally by humans ([Eggink et al., 2020](#)). Nevertheless, this particular avian IAV demonstrates the volatility of the barrier between poultry and humans. Changes in amino acids not directly involved in contact with SAs also affect anchoring contact for SA ([Sriwilajaroen and Suzuki, 2012](#)).

Anatomical distribution of SAs helps to determine the transmissibility of IAVs, a factor involved in host ranges and influenza pandemics ([Xiong et al., 2014](#)). SAs found in the respiratory tract of the pig are observed both in humans and chickens; because of the distribution of these SAs along the epithelia of the pig's respiratory tract, they are considered to be mixing vessels for IAVs, leading to new viral strains with the potential to infect humans. Large-scale pig farming is also subject to many of the issues we have raised about poultry production ([Robinson et al., 2014](#)) and add to the complexity of changes of host range in circulating IAVs.

## THE ROLE OF THE MHC IN ESTABLISHING SELECTIVE PRESSURES UPON IAVS MEDIATED BY ACQUIRED IMMUNITY

### Antibodies and MHC class II

Antibodies generate protective immunity against IAV ([Kosik and Yewdell, 2019](#)) and, consequently, they are able to drive changes in host ranges and create a zoonotic potential for IAVs (see, e.g., [Peacock et al., 2017a, 2017b](#); [Wang et al., 2021](#)). However, although we argue that low variability at the MHC of commercial poultry can be a driving force behind changes in the host range of IAV, it is unlikely that low variability of MHC class II will be a mechanism behind this switch. This is because there will most likely always be a ligand of HA and NA-derived peptides for any of the avian MHC class II alleles that, in the case of viral infections, do not participate directly in the specificity of antibody effector mechanisms *per se*. Indeed, HA mutants can escape recognition by specific CD4<sup>+</sup>, but are still neutralized by antibodies ([Berkhoff et al., 2007](#)).



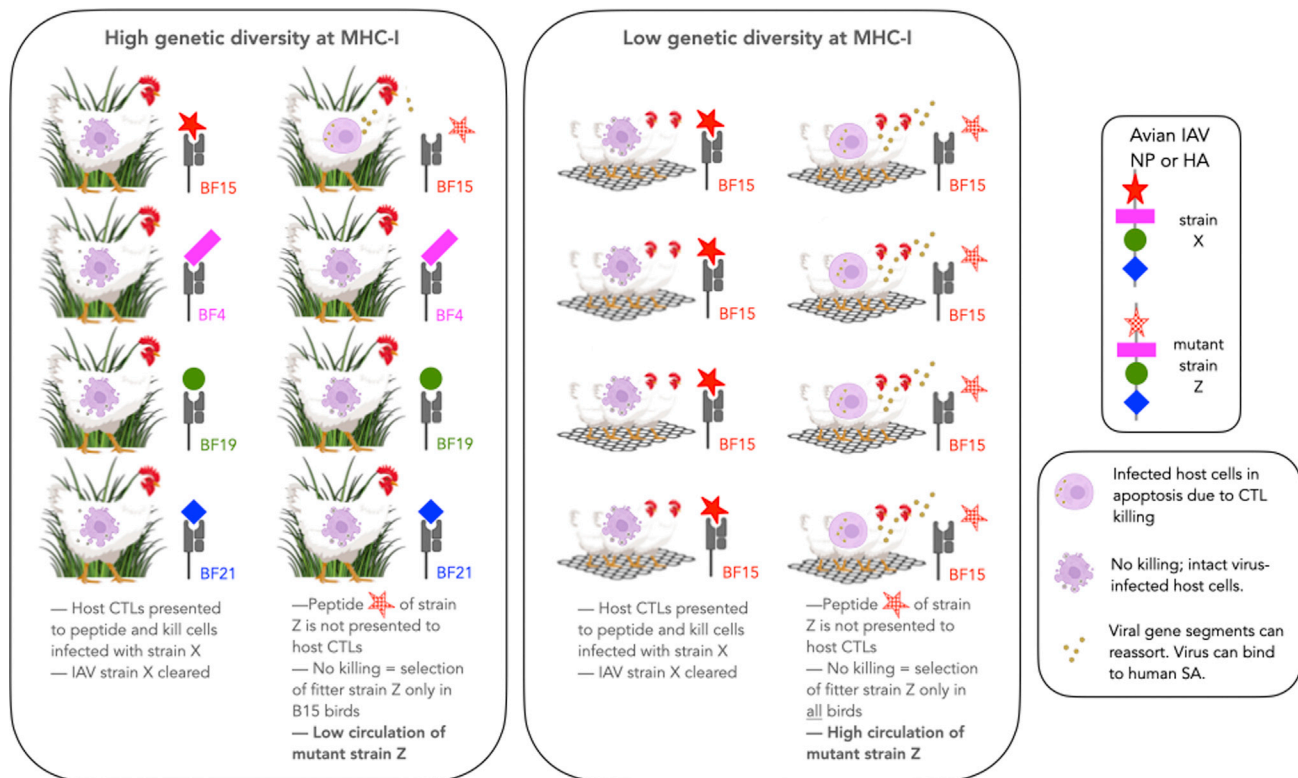
### CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) and MHC class I

Immunity to IAV delivered by CTLs, differently from neutralizing antibodies, does not prevent infection, but kills cells hosting replicating viruses. CTL activity is restricted by MHC class I. If processing by host proteasomes of a mutated viral protein involved in species-specificities of IAV structures such as SAs, MxA, etc., results in peptides that are unable to bind to a Class I BF2 allele to be presented to CTLs, the virus-infected cell bearing the allele(s) will not be killed. Such a mutant virus will be fitter because the cells that it infects are not recognized by its host's CTLs. Thus, low variability in this locus is expected to be a strong pressure for the selection of escape mutants, including those with altered host specificities. A viral variant that escapes antibodies will have a selective advantage when infecting numerous other individuals with humoral immunity to wild-type strains. Conversely, if CTL responses are restricted by MHC class I in a population that is highly polymorphic, IAV will gain a selective advantage in only a small fraction of hosts (Berkhoff et al., 2007b; Thyagarajan and Bloom, 2014). However, in a population of thousands of broilers with few BF2 alleles, CTL-mediated selective pressure might be as strong as antibody-mediated selection. In a population presenting low diversity in MHC class I loci, the chances for a single fit, escape mutant to appear in many hosts are thus greater (Figure 2). Concerning the hypothesis addressed herein, the following questions must be answered: is there evidence that CTLs exert selective pressures upon avian IAV proteins? Is there evidence that mutations lead to changes in proteins involved in species-specificities? Studies on the role of CD8<sup>+</sup> CTL-mediated immunity against such proteins indicate that it can indeed drive immune escape in targeted T cell epitopes (reviewed by La Gruta and Turner, 2014); therefore, the consequence of these mutations affecting the host range is a possibility.

CTLs that are cross-reactive for IAV strains are usually specific for the less variable viral proteins (i.e., NP, M proteins, and polymerases) in contrast to HA and NA because of the former proteins' higher levels of functional constraints (Li et al., 2019; Visser et al., 2016). However, comparing sequences of IAV isolated over several decades indicates that even conserved proteins such as NP present a high frequency of mutations. Importantly, they result in loss of CD8<sup>+</sup> CTL epitopes (Berkhoff et al., 2007a). Whereas strain-specific immunity tends to be directed toward HA, which is more tolerant of mutations (Thyagarajan and Bloom, 2014; Visser et al., 2016), NP can present CTL-escape mutants that have been selected during actual infections in humans during seasonal flu outbreaks (Rimmelzwaan et al., 2004). Furthermore, CTL-escape mutants emerged in an immunocompromised patient with an antibody deficiency who presented a prolonged infection with IAV; this phenomenon was also observed in a model with immune-deficient mice (Valkenburg et al., 2013).

Mutations in NP affect the packaging efficiency of IAVs' segmented genome and, consequently, viral fitness (Brooke et al., 2014). Host range can be affected by a single amino acid substitution in an NP: the mouse IAV A/Puerto Rico/8/1934 (H1N1) strain presented reduced expression of mRNA encoding NA and vRNA; this substitution affected packaging, generating non-infectious virions lacking gene segments that outnumbered intact virions. Of relevance for our hypothesis, the NA segment is the most frequently absent in defective packaging. Non-infectious virions are rescued by recombination, which occurs at high frequency in animal models, and produce infectious progeny; in the aforementioned H1N1 strain, the NP mutation resulted in its adaptation from mice to guinea pigs (Kosik and Yewdell, 2019). Therefore, the selection of an epitope of NP by CTLs can also indirectly select mutant NAs by increasing the packaging of recombinants. In addition to this event, the lack of immunity to a recombined virus that changed its host range will give it an advantage over circulating seasonal strains to which many humans may be immune. Thus, NP mutants can increase reassortment events between mutant HAs and NAs and affect IAVs' host range indirectly. We have already discussed above how mutations in IAV NPs can mediate escape from restriction by human MxA proteins and enable infection of avian-like swine IAVs in human cells (Dornfeld et al., 2019). NP mutants thus could be selected in large populations of chickens presenting a single or few MHC alleles.

An HLA-B27-restricted CTL escape mutant on NP, which lost the anchor residue for this HLA allele, emerged in the 1993–1994 influenza season. Although this viral strain would seemingly have an advantage in only about 8% of the population (Marsh et al., 2000) (i.e., the bearers of the HLA allele that no longer presented any NP epitopes), it was fixed and persisted in human populations for several influenza seasons. Modeling exercises indicate that this mutation was advantageous for the virus because it conferred longer infection periods in hosts and, thus, greater chances of surviving until the next influenza season (Gog et al., 2003). Characteristics of seasonal influenza outbreaks observed in humans may not apply to commercial



**Figure 2. Mechanism for selection by reduced MHC diversity of avian IAV mutants with potential for increased virulence and/or inter-species spillovers**

A schematic representation of NPs or HAs in two strains of avian IAVs depicts possible peptides (colored forms) for presentation to CTLs within distinct peptide binding grooves (PBGs) encoded by different alleles of chicken BF MHC class I. In a situation of high genetic diversity at BF (depicted in the two columns of birds to the left of Figure 2), CTLs will kill host cells infected with IAV strain X and clear the virus, regardless of the viral NP or HA peptide presented; cells from BF15 birds infected with escape mutant Z are not killed because they are unable to present any NP or HA-derived peptides to CTLs. Mutant Z is thus selected, but circulates in a small number of birds and presents a lower chance for inter-species spillovers (e.g., to humans working at industrial farms) or for mixing if infected birds reach live poultry markets. In a situation of low genetic diversity (the two columns of birds to the right of Figure 2), cells from BF15 birds infected with escape mutant Z will be not killed. As in a situation of high genetic diversity at BF, mutant Z is thus also selected, but it now circulates in a large number of birds, thus presenting a higher chance for inter-species spillovers or for reassortment of viral gene segments and mixing with other birds in live poultry markets. In this example, the following codes and premises apply: peptides of HAs colored in red in strains X and mutant Z contain amino acids located at the protein's RBS; the RBS of mutant strain Z's HA presents ability to bind to human SA; mutant strain Z's red peptide lost the ability to bind to BF15' PBG; mutant strain Z's NP or HA proteins do not suffer functional constraints and the selected virus is fit. For clarity, molecular interactions between MHC peptide-binding grooves and amino acids of viral HA peptides are simplified and four alleles represent a situation of high genetic diversity and one allele represents a situation of low genetic diversity in two flocks of thousands of birds each. Also, for clarity, the example resorts to mutants at NP's and HA's SA-binding RBS, but all mutations that affect viral proteins involved in species-specificities of IAV, e.g., example cleavage sites of HA by different proteases are subject to the same mechanisms of selection. The figure was created with MindGraph

poultry flocks; nevertheless, this study illustrates the biological consequences of CTL-mediated selection of viral strains by very frequent MHC class I alleles. Adding to this complexity, epistasis between HA and NA may complement antigenic drift in these proteins, and, thus, the binding specificity and host range of a new viral strain. This is shown by the effect that monoclonal antibodies specific for HA have in rescuing and co-selecting NA mutants (Kosik and Yewdell, 2019).

IAV's M protein determines the host range in a more direct way. If an avian IAV infects mammalian cells, excessive splicing of its M protein's mRNA7 occurs and this causes viral interference in the mammalian host cells, blocking cellular processes that the virus would otherwise use to complete replication and budding. However, mutagenesis experiments of the M protein show that non-synonymous changes of amino acids in certain regions modulate the efficiency of mRNA7 splicing (Calderon et al., 2019). It is reasonable, therefore, to speculate that avian CTLs from a population presenting few MHC class I alleles may select for mutants of the M protein that affect splicing of gene segment 7 that encodes for M protein

in a way that renders it compatible with mammalian cellular processes. Other viral proteins involved in host specificity, such as NS1, have also been shown to induce CTLs (Man et al., 1995) and thus are subject to the same premises of our arguments.

Regarding HA and NA, pivotal proteins in the species-specificity of IAV, both can be recognized by CD8<sup>+</sup> CTLs (Bennink et al., 1984; Bodewes et al., 2010; Braciale et al., 1989; Ennis et al., 1977; Goy et al., 2008; Jameson et al., 1998; Kosor Krnic et al., 2008; Sweetser et al., 1989; Townsend et al., 1986;; ; Wysocka and Hackett, 1990). However, target epitopes of HA and NA are less frequent when compared with those present in viral internal proteins. This reflects the immunodominance hierarchies of the viral proteins and the number of CTL MHC class I epitopes available in each. Compared to the M, NP, and PB1 proteins, there are fewer CTL epitopes in HA and NA when naturally infected humans were studied (Lee et al., 2008). This fact might reflect the different abundances of the viral proteins in the cytosol (reviewed by Bodewes and colleagues, 2010). Furthermore, these HA and NA epitopes may not be part of the RBS of these proteins. However, immunity to the antigenically conserved stem region of viral HA affects the activity of neuraminidase and thus, potentially, the host range. This modulatory event is thought to be dependent on stem-specific antibodies (Kosik et al., 2019). Nevertheless, escape mutants of the HA stem could be generated by CTLs and could render antibodies more or less effective in modulating NA functions.

The last argument for a role of low levels of polymorphism in MHC class I alleles in affecting the host ranges of avian IAVs is the fact that when CTLs target and kill IAV-infected cells they also reduce B cell responses by eliminating antigens that would otherwise be priming CD4<sup>+</sup> T helper cells (Webby et al., 2003). Therefore, it is reasonable to speculate that CTL-escape mutants of avian IAV could provide a stronger antibody response for avian hosts and, consequently, correspondingly stronger selective pressures on HA and/or NA that result in substitutions of critical amino acid residues in their RBS, resulting in a new host range.

Whereas the protective role of CTLs against human IAVs and in selecting mutants of IAV proteins is established, there are few studies examining these same aspects in chickens. CD8<sup>+</sup> CTLs do participate in protective immunity against a lethal infection with a strain of HPAIV (Seo et al., 2002) and CTLs also mediate cross-protective immunity for distinct strains of avian IAVs (Seo and Webster, 2001). Some studies have also observed an association between chicken lines presenting distinct alleles of BF2 and challenged with LPAIV and levels of MHC class I expression on antigen-presenting cells and macrophage, B cell, and T cell frequencies in infected tissues (Aston et al., 2021). Another study observed that macrophages from disease-resistant chickens of the B2 haplotype are more efficient at activating T lymphocytes than macrophages from disease-susceptible, B19 haplotype chickens (Collisson et al., 2017). Regarding the role of different BF2 alleles in responsiveness to specific IAV proteins, studies are also limited. Hou et al. (2012) immunized four common chicken class I haplotypes with an avian IAV NP and identified NP CTL epitopes only in chickens of certain haplotypes among those tested. Reemers et al. (2012) infected chickens of five haplotypes with IAV, stimulated their lymphocytes with dozens of peptides derived from NP and M1 proteins and identified 12 MHC B12-restricted, 3 B4-restricted, and 1 B19-restricted CD8<sup>+</sup> T cell epitopes from these proteins among the haplotypes examined. Interestingly, no epitopes were identified for the promiscuous B21 allele (Kaufman, 2018). These results show that for the few proteins studied, some BF2 alleles did not present epitopes derived from NP or M1 to host CD8<sup>+</sup> T cells. So far, there is one report for a CD8<sup>+</sup> T cell epitope, the 15-mer peptide H5246–260, within the HA of an avian H5 AIV and it induced the expression of granzyme A by CTLs of the genetically defined line P2a of chickens (homozygous for the B19 haplotype) immunized with this HA (Haghighi et al., 2009). A BF2\*15-restricted CTL epitope, peptide NP67–74, has also been described for the NP protein of H5N1 IAV in chickens (Zhang et al., 2016). Li et al. (2020) developed a screening assay for IAV-derived peptides bound to BF2 grooves. Stable CTL epitopes for the high-expressing BF2\*1501 were identified *in silico* for four strains of the H5N1 HPAIV: HA, NP, PA, and PB1; M2 protein did not present stable epitopes for this haplotype. Further alignments with 203 additional strains of H5N1 reported in China between 1996 and 2016 were performed and confirmed the high conservation of these peptides within these proteins, except, interestingly, for HA.

## CONSEQUENCES OF LOW MHC VARIABILITY IN THE OUTCOME OF IAV INFECTIONS IN POULTRY

Current poultry breeding and production systems may provide selective pressures toward IAV variants with elevated virulence for poultry itself (e.g., by generating a receptor specific for SAs of the tissues where viral replication and transmissibility takes place). In poultry, MHC class I genotype is found to be associated with

resistance or susceptibility to IAV, depending on breed and genetic line studied. After an outbreak of H5N1 avian IAV, among 340 surviving and 390 dead Thai indigenous chickens from smallholder farms, all birds presenting with the homozygous B21 haplotype were survivors and heterozygous B21 animals presented a high survival rate; on the other hand, among dead birds, 100% were homozygous for B13 haplotype (Boonyanuwat et al., 2006). When much smaller numbers (10 in each line) of MHC congenic white leghorn chickens were challenged with HPAI H5N1 IAV, the same trend was observed, although with weaker associations of outcomes with these two haplotypes (Hunt et al., 2010). Matsuu et al. (2016), however, did not find a significant association between MHC BF2 class I polymorphisms and resistance to HPAI H5N1 IAV in white leghorn chickens. Associations of MHC haplotypes with resistance and susceptibility to infectious diseases are much stronger in chickens than in humans (Miller and Taylor, 2016). For this very reason, if there is low variability in these haplotypes in a given commercial population owing to breeding practices (Márquez et al., 2010), pressure may increase upon viruses to escape immune responses by avoiding binding to MHC molecules.

Avian influenza viruses can spontaneously mutate to highly pathogenic variants, causing high mortality in poultry (Beerens et al., 2020). Pathogenicity in avian IAVs can be determined by the amino acid sequence of the site within HA that contains the fusion peptide for host cells. The sequence determines susceptibility for cleavage of HA by different proteases. When it contains a single basic amino acid, arginine, it is cleaved by host serine proteases of limited distribution and is, correspondingly, a strain of low pathogenicity. Conversely, if it contains multiple basic residues (arginine or lysine), the so-called polybasic cleavage site, it will be cleaved by furin, a protease with a much wider distribution in the host, explaining the wider tissue tropism and, thus, the higher pathogenicity of such IAVs (Böttcher-Friebertshäuser et al., 2013; Braun and Sauter, 2019). The polybasic cleavage site is a prime determinant of virulence for avian IAVs (Horimoto and Kawakita, 1994). To the best of our knowledge, nothing is known about the role of MHC genetic variability in this phenomenon. Epidemiological evidence indicates that HPAIVs arise mainly in intensively farmed birds (Alders et al., 2014), raising the possibility that low diversity of MHC class I may play a role in this phenomenon. However, at least in one instance, HPAIVs may have arisen from wild swans in Iran (Gilsdorf et al., 2006). Since bottlenecks frequently occur that affect the genetic diversity of wildlife, including at the MHC, the question then arises: what was the level of diversity of class I MHC in the reservoir population of wild swans? This is not known; in addition, the size of swan populations in Western and Central Asia is considered by conservationists to be uncertain (Rees et al., 2019).

Herd immunity of the acquired type, dependent on antibodies and CTLs, is known to drive antigenic drift of influenza viruses (Webster et al., 1992). Some studies have determined that vaccination promotes evolution and antigenic drift of avian and human influenza viruses (Boni et al., 2006; Lee et al., 2004). On the flip side, the survival of certain chicken haplotypes after an outbreak of avian influenza has also been observed: outbreaks of H5N1 avian influenza virus in 2004–2005 caused human deaths and devastated poultry industries in Asia. However, as already reported above, chickens on small farms in Thailand, where there are mixes of genes from commercial and indigenous breeds, weathered the outbreaks well, depending on their haplotypes of MHC (Boonyanuwat et al., 2006). Interestingly, the majority of birds homozygous or heterozygous for the B21 allele, which is promiscuous in terms of peptides presented to the immune system (Kaufman, 2018), were in the group that survived. Conversely, birds homozygous for the fastidious peptide-presenting B13 allele had a nearly 100% mortality rate. Indigenous chicken breeds from Southeast Asia possibly carry larger repertoires of polymorphisms for genes encoding components of the immune system because that region is the center of origin of chickens and domestication events (Miao et al., 2013).

One study examined the possibility of selecting viral mutants in populations of commercial birds presenting very low variability of antigen-presenting MHC haplotypes. Semicongenic chicken lines genetically resistant (MHC-B21) and susceptible (MHC-B13) to Marek's Disease were vaccinated against the disease with a cloned virus to examine the evolution of the pathogen during the infection. The study found that *in vivo* passaging of the Marek's Disease virus in this model, involving both host genetics and vaccination, was selected for increased viral virulence (Hunt and Dunn, 2015).

PB1-F2 and N40 of IAV are encoded in the same gene segment encoding the PB1 subunit of the viral trimeric polymerase; whereas their genetic variants generally do not affect host range, they are associated with or cause different levels of pathogenesis and of growth of IAV (Vasin et al., 2014; Wise et al., 2009) and one study showed that they even may affect its host range (Gíria and Rebelo de Andrade, 2014). Variants for

these genes may be selected by MHC class I. If this selection occurs in a population of poultry with low diversity at class I, this could result in a virus with increased virulence. Experiments assessing transmission dynamics of avian influenza viruses between wild waterfowl and commercial chicken and turkey lines observed changes in the polymerase and accessory genes of the pathogenic H5N8 virus and also high mortality typical of highly pathogenic variants when the virus changed hosts (Puranik et al., 2020). Experiments to assess selective pressures upon IAVs by poultry MHC haplotypes have not been performed.

In all, the small effective population size of commercial poultry lines caused by breeding programs for productivity traits may be self-defeating if reduced variability of immune response genes renders animals more susceptible to devastating viral infections or even selects for more virulent avian pathogens.

### STRATEGIES TO EVALUATE THE ROLE OF LOW GENETIC VARIABILITY IN POULTRY IN THE EVOLUTION OF AVIAN IAV

Although it is important for testing the hypothesis presented in this review, there is little information about MHC class I-restricted epitopes of AIV recognized by chicken CD8<sup>+</sup> T cells. Peptidomics could facilitate obtaining this information for testing predictions about selective pressures mediated by populations of animals presenting very low frequencies of MHC class I alleles. Experiments *in vivo* to test the hypothesis set forth in this review are possible, but difficult to perform owing to safety concerns and logistics. Studies *in vitro* are safer and easier and, in order to be substituted by the even more expeditious *in silico* approaches, they first must be undertaken in order to build the databases on which predictive algorithms are trained. In many cases, *in silico* predictions of IAV-derived peptides for human class I alleles have not been confirmed by *in vitro* studies (reviewed in Wu et al., 2011). This is probably also true for predicting epitopes from avian IAVs for the chicken MHC because such studies in this species are incipient. To the best of our knowledge algorithms have not been created for avian MHCs; however, full-length sequences can be uploaded and used to obtain predictions for any custom MHC class I molecule at NetMHCpan - 4.1 (<https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1>) of the Technical University of Denmark (DTU).

An immediate approach with peptidomics of BF2 in the chicken-IAV interface would be to examine epitopes eluted from IAV-infected cells from BF2-typed chickens or BF2-transfected cell lines expressing frequent and rare class I alleles and confront them with the sequences of the viral proteins and respective domains involved in host range and virulence discussed in this essay in representative influenza isolates. However, before undertaking systematic analyses for discovering BF2-dependent CD8<sup>+</sup> T cell epitopes derived from IAV it would be useful to genotype representative samples of commercial chicken flocks formed by different breeds and or purebred and crossbred genetic lines in order to determine the types and frequencies of alleles present in large populations of poultry in actual commercial production settings. Then these alleles can be used in peptidomics experiments to transfect cell lines. BF2-transfected cell lines can then be infected with relevant strains of avian HPAIVs and LPAIVs to see what peptides are being presented and how they align to different viral strains. There is an extensive database of sequences of IAV proteins from numerous isolates against which these peptides can be aligned (as per experiments described by Li et al., 2020) in order to make inferences about mutations and consequences for species-spillovers. Samples of cells from animals from commercial flocks dying of and surviving outbreaks of avian influenza should be studied to make correlations of outcomes with BF2 alleles and must also undergo peptidomics studies; to the best of our knowledge, this approach has not yet been undertaken. Reagents against specific BF2 alleles will be needed.

For human diseases, similar *in vitro* approaches have been applied to HIV (Arora et al., 2019). A peptidome-wide association study (PepWAS) examined the HLA genotypes and set-point viral loads of 6,311 AIDS patients. The set-points indicate when CTLs are functional in controlling the virus and are significantly associated with certain HLA alleles. The HIV proteome eluted from patient HLA-A and-B proteins generated 3,252 unique peptides, some of which enable predictions for the set-points. Relevant peptides have also been identified from peptidomes obtained from HLA and MaMu alleles of humans and rhesus macaques, respectively, that are elite controllers of HIV and SIV (Marcilla et al., 2016).

### STRATEGIES FOR AVOIDING SELECTIVE PRESSURES FROM LIVESTOCK ON VIRUSES

Technologies such as genomic selection accelerate genetic gains for animal productivity and bring dramatic improvements in desirable production traits, especially when measurements can only be performed

in one sex (milk, eggs), after slaughter (meat tenderness), or at older ages (stability, which measures the length of a cow's productive life in a herd) (Boichard et al., 2015; García-Ruiz et al., 2016). In dairy cattle, gains in genetic progress resulting from the wide adoption of these new technologies have been so significant adjustments in selection objectives became necessary to maintain gains in production traits, while inverting negative trends in eroding traits (García-Ruiz et al., 2016). Genomic selection has also been adopted by poultry and swine breeding industries, with similar impacts (Misztal et al., 2020). Practices to maximize heterosis are also widely adopted (Iversen et al., 2019). The inclusion of new traits related to the level of immune system polymorphisms could be achieved by breeders at low or no additional direct costs by supplementing already existing genotyping chips with additional content, with important tangible and intangible positive impacts on productivity and consumer attitudes, respectively.

The costs involved in fine-tuning current breeding strategies to provide greater variability at the avian MHC in commercial production populations pale in comparison to the economic losses incurred by influenza in humans as DALY (disability adjusted life years). In the European Union, of the estimated 1.38 million DALYs for all infectious diseases for the period between 2009 and 2013, influenza had the highest burden, representing 30% of this total (Cassini et al., 2018). In 2017, the WHO estimated that seasonal flu causes up to 650,000 deaths each year (Lee et al., 2018) and estimates from 2015 compute average losses caused by influenza in the United States to be \$11.2 billion (\$6.3–\$25.3 billion) (Putri et al., 2018). In addition, if our hypotheses are correct, increased MHC variability may directly contribute to increasing poultry disease resistance, and therefore generate direct economic gains to breeders and farmers. When considered altogether, direct economic gains added to potential gains from consumer perceptions of less tangible benefits to human health may comprise considerable opportunities for animal protein production industries, especially poultry and swine, to benefit from.

## CONCLUSIONS

Pathogens adapt to modern interventions used to treat and prevent disease by evolving resistance to antimicrobials (World Health Organization, 2016a, 2016b), and vaccines (Andam and Hanage, 2015); more virulent pathogens can also evolve under these selective pressures (Cepas and Soto, 2020; Read et al., 2015). Humans present high levels of genetic diversity in MHC proteins, and, consequently, are able to react to changes in genes encoding the polymerase complex and antigenic regions of hemagglutinin of an influenza virus, and replace it with other clades (Memoli et al., 2009). These facts underscore the resulting pressures presented by the low levels of variability in MHC observed in commercial chicken lines and by management practices that suppress immunity, leaving one or few avian MHC class I alleles as the sole selective pressure for avian IAV in large cohorts of poultry. More data are needed on the impact of low MHC diversity of livestock for selecting pathogen virulence and species-specificities to challenge these hypotheses. Furthermore, data on MHC diversity is needed for all animals, whether wild-life or domesticated animals, in natural environments or bred in captivity and in production systems for food. Population bottlenecks have several origins, frequently occur, and affect the genetic diversity of species, including at the MHC and many animals are reservoirs of zoonoses.

The impacts of antibiotics as growth promoters for livestock, animal welfare, balanced ecosystems, levels of consumption of animal protein, and cultural preferences for consuming threatened wildlife, which can present low MHC variability (Castro-Prieto et al., 2011, 2012; Radwan et al., 2010, 2020), can be pivotal in new pandemics. Ironically, low genetic variability is not the only type of uniformity that might be detrimental to poultry production: monopsony of the market structure for producing poultry meat has been criticized as a cause of income inequality, economic stagnation, and lack of innovation, among other evils (Lowry, 2018). As aptly argued by Wallace (2009), influenza pandemics also have social origins. Education of consumers about the consequences of these aspects of food production is an important force in changing disease ecology, and in a more promising future, a good balance between industry, consumers, and society in general will be reached, equating the economic, social, environmental, and scientific interests.

## LIMITATIONS OF STUDY

This review raises the hypothesis that animal husbandry strategies for producing inexpensive animal protein for food have a significant potential to render selective pressures on avian influenza viruses to become zoonotic or highly pathogenic. Observational and experimental data that *directly* support this hypothesis is not yet abundant. The purpose of the review is to raise interest in undertaking research actions on this issue.

## SEARCH STRATEGY AND SELECTION CRITERIA FOR THIS REVIEW

References for this review were identified through searches of PubMed and Google by use of the terms "avian influenza A virus," "host range," "MHC," "genetic variability," "selective breeding," "stress," "immunity," "meat," "human evolution," "hemmagglutinin," "neuraminidase," "host specificity," "furin," "nuclear protein," "matrix protein," "non-structural protein," "endosomal pH." Articles resulting from these searches and relevant references cited in those articles were reviewed.

## STUDY CONCEPT AND DESIGN

I.K.F.M.S. conceived the hypothesis addressed in this review, and M.M.C., A.R.C., and I.K.F.M.S. contributed equally to all aspects of the arguments supporting it and wrote and reviewed the manuscript.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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