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Different breeds, different blood: Cytometric analysis of whole blood cellular composition in chicken breeds

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ABSTRACT

While haematological variation is well known in birds, variation in avian breeds (distinct morphotypes of the same species) remains unexplored. Poultry breeds, in particular, may show interesting evolutionary patterns and economically-relevant physiological differences. We performed a comparative examination of blood cellular composition in five chicken (*Gallus gallus domesticus*) breeds: Araucana, Booted bantam, Czech, Minorca and Rosecomb bantam. In standard-environment-reared hens whole-blood flow cytometry revealed remarkable differences in most erythrocyte- and leukocyte-related parameters. We identified two extremes: Czech, a European breed, with a low heterophil/lymphocyte (H/L) ratio and high $CD4^+$ levels, and Araucana, a South-American breed, with a high H/L ratio and high relative monocyte count. Such variation may reflect a combination of artificial and natural selection acting on health- and stress-related traits in domestic populations. Different breeds have evolved different immunological adaptations reflecting their original need to fight pathogens and physiological constraint resulting from dissimilar physiological trade-offs.

1. Introduction

Evolution has shaped the immune system of each animal species slightly differently in response to distinct selective pressures. This is also reflected in species-specific blood cellular composition. Interspecific haematological differences are well known for birds and several attempts have been made to provide an evolutionary explanation for this (Blount et al., 2003). While there has been much work at the species level, little attention has been paid to population level studies, and to traditional breeds (i.e. artificially selected morphotypes of the same species) in particular. The domestic chicken (*Gallus gallus domesticus*) in which most breeds have been created, is a species with enormous agricultural and scientific value (Burt and White, 2007). Most present research on chickens is conducted using a limited number of chicken lines only that generally share a very recent common origin (Delany and O'Hare, 2008). However, contemporary chicken populations are highly diverse (Hillel et al., 2003) and this variability may have both academic and commercial importance (Wright et al., 2009). There are currently hundreds of distinct chicken breeds displaying a wide range of phenotypic traits (Rubin et al., 2010) which have been specifically selected for over a millennia-long domestication process. While production traits and variation in exterior appearance are conspicuous in these breeds (Halima et al., 2007; Hocking et al., 2003; Wright et al., 2009), it is also highly likely that they will display substantial immunological variation.

The enormous genetic and phenotypic variability in chicken breeds originates from the unique evolutionary history of their domestication process. Modern chickens most likely arose from four different geographically separated subspecies of red junglefowl (*Gallus gallus gallus, G. g. spadiceus*, *G. g. jaboullei* and *G. g. murgha*; Hillel et al., 2003; Kanginakudru et al., 2008; Liu et al., 2006; Miao et al., 2013) through multiple domestication events (Kanginakudru et al., 2008; Liu et al., 2006). Possible admixture of the grey junglefowl (*Gallus sonnerati*) was also suggested (Eriksson et al., 2008). The polyphyletic origin of the domestic chicken, together with later artificial selection, has resulted in an initial inter-breed variation level comparable with that resulting from interspecific variation in free-living species. Intriguingly, it is highly likely that natural selection has shaped and diversified functional variation in different ways as a result of differences in the natural conditions and pathogenic pressures occurring in the different regions where chickens have been bred. This opens the possibility that chicken breeds, which have been generally overlooked in contemporary re-

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search, harbour immunological variation that may be missing in laboratory research lines and commercial strains. Thus, traditional chicken breeds may prove highly important for further improvements in commercial chicken health and biosecurity.

Haematological assessment of blood cellular composition is a basic requirement for descriptions of cell-mediated immune function (Beaufrere et al., 2013; Fair et al., 2008; Gottstein et al., 2015; Iseri and Klasing, 2013). In previous studies on birds, absolute and relative blood cell counts have commonly been used as diagnostic markers of health- or stress-state (Davis et al., 2008; Pickler et al., 2013; Talebi et al., 2005). The small number of studies thus far performed on African and Asian chicken breeds/morphotypes have suggested differences that may exist in basic haematological parameters between breeds (Islam et al., 2004; Peters et al., 2011). However, these studies were performed using light microscopy that, given the relatively small sample size of the cells typically investigated, may provide relatively unprecise estimates and does not allow for more advanced comparison of individual breeds. Recent methodological advances have made flow cytometric analysis with fluorescent-labelled antibodies the state-of-the-art method for haematological examination of blood (Beirao et al., 2012). Unfortunately, unlike mammalian blood, bird blood contains nucleated erythrocytes and thrombocytes, which complicate analysis of whole blood samples (Beaufrere et al., 2013; Sellier et al., 2007). In most studies, therefore, leukocytes have been separated for analysis (Bridle et al., 2006; De Boever et al., 2010; Fair et al., 2008; Chen et al., 2012), though this leads to cell-loss during the isolation process. Alternatively, erythrocytes have been lysed using buffers with unknown impact on the different cell types (De Boever et al., 2010). Recently, Seliger et al. (2012) designed a novel no-lyse technique allowing flow cytometric cell quantification of whole blood without the necessity of leukocyte separation.

In response to these methodological advances, we performed a no-lyse flow cytometric whole blood analysis of five traditional chicken breeds in order to describe their haematological cellular composition, thereby providing basic data for analysis of breed-specific immune adaptations resulting from distinct evolutionary histories. While haematological parameters are partially genetically determined, there is also a very strong environmental effect on these traits (Bridle et al., 2006; Chen et al., 2012). In order to control for external rearing effects, all subjects were maintained under the same standardised environmental conditions from incubation until adulthood when the blood composition was analysed. In order to verify the reliability of our method, we also compared our cytometric results with those obtained using microscopic examination of blood smears. By showing remarkable immunological variation in traditional chicken breeds we highlight that the breeds may show evolutionary adaptations with potential for practical application.

2. Methods

2.1. Animals

We obtained 535 presumably fertilised eggs of five chicken breeds (Araucana, Booted bantam, Czech (Czech golden pencilled ~ Czech golden brindled), Minorca, and Rosecomb bantam) from small non-commercial breeders in the Czech Republic, EU. All eggs were individually inscribed on the shell with the breed, breeder and starting date of incubation. The eggs were incubated in an OvaEasy 380 Advance EX automatic egg incubator (Brinsea, Weston-super-Mare, UK) at a temperature of 37.5 °C and 50% humidity. Eggs of the same origin (same breed and breeder) were kept together in separate enclosed trays inside the incubator in order to prevent any mismatch in unmarked newly hatched chicks. We always started incubation of eggs of the same origin on the same day, resulting in the same hatching date. All chickens within this experiment hatched between 13th May and 22nd June 2015. All hatchlings were individually marked with two numbered wing-markers (one mark on each wing) before being moved from the incubator to cages in the animal facility (Czech University of Life Sciences Prague). Groups of three to seven chickens were housed in cages (0.50 width × 0.88 depth × 0.45 m height) according to origin with access to food and water *ad libitum* (standard wheat-based feed mixture; Sehnoutek a synové s.r.o., Czech republic, EU; for complete feed composition see Table S1). For the first week after hatching, chicks were kept at room temperature (32 ± 2 °C) and then were gradually adapted to final temperature of 20 ± 2 °C. No bird received vaccination. Individual origin groups were distributed around the animal facility at random. Sex was determined based on both genetic and morphological markers. Genetic sex determination was performed according to Griffiths et al. (1998), using a drop of blood from the wing vein. Ninety-nine females representing five breeds and 17 breeders, with 1–31 individuals per breeder, were selected for further investigation (Table S2). Those females selected were rehoused at one to two individuals per cage (only chickens of the same origin together) and again randomly distributed around the animal facility. In the time of haematological analysis, all individuals were adult (age ranging from 29 to 35 weeks) and did not show any signs of illness. This research was approved by the Ethical Committee of the Faculty of Science, Charles University (Reference no. 1373/2016-4).

2.2. Antibodies

Four antibodies were used to differentiate individual blood cell types in the test subjects (Table 1). Antibodies Anti-CD45-PE, Anti-CD4-Alexa 700, and Anti-macrophages-FITC (KUL01) were obtained from SouthernBiotech (Birmingham, USA), while Anti-CD51/61-Alexa 647 was obtained from BioLegend (SanDiego, USA). The antibody mixture used for blood sample staining contained 8 µL (0.5 µg/µL) KUL01, 2 µg (0.5 µg/µL) Anti-CD45-PE, 1.5 µL (0.5 µg/µL) Anti-CD4-Alexa 700, and 3 µL (0.2 µg/µL) Anti-CD51/61-Alexa 647 per 50 µL sample total volume. After titration trials, all antibodies were used in concentrations recommended by the manufacturer.

2.3. Flow cytometry

During flow cytometric analysis, we followed the method described previously by Seliger et al. (2012), with several modifications. To minimise animal handling time as well as any other experimenter-mediated effects on the haematological traits, a blood sample was collected right after an individual was taken out of its cage. Each whole blood sample was collected from the brachial vein using a heparin-treated syringe and kept on ice until analysed. Although heparin-treated samples provide slightly different results for thrombocytes than

Table 1
List of antibodies used for differentiation of individual blood cell types.

Name	Specificity	Clone	Fluorochrome	Isotype	Cells stained	Reference
Anti-CD45-PE	chCD45	CT40	PE	Mouse (BALB/c) IgMκ	Leukocytes, thrombocytes	Schultz and Magor (2014)
Anti-CD4-Alexa 700	chCD4	CT4	Alexa 700	Mouse (BALB/c) IgG1κ	CD4+ T lymphocytes	Chan et al. (1988)
KUL-01-FITC	chMRC1 homologue	KUL-01	FITC	Mouse (BALB/c) IgG1κ	Monocytes, macrophages	Staines et al. (2014)
Anti-CD51/61-Alexa 647	hCD51/61	23C6	Alexa 647	Isotype Mouse IgG1κ	Thrombocytes	Viertlboeck and Gobel (2007)