Expanded Materials and Methods

Hemagglutination/Hemolysis Analysis

I assessed innate humoral immunity by using a hemolysis-hemagglutination (HL-HA) assay to characterize natural antibody (NAb)-mediated agglutination and lysis of exogenous red blood cells (RBCs) as described by Matson et al. (2005). This HL-HA assay requires a 50-µL sample of blood plasma per individual, which is serially diluted along the long axis of a 96-well microtiter plate. Diluted plasma samples are incubated with exogenous RBCs. Assays were randomized and run blindly with respect to sample; digitized images produced by scanning plates at assay completion were randomized with respect to plate, plate location, and sample, and were scored blindly for both maximum lytic activity and agglutination. Both lysis and agglutination are recorded as the negative $\log_2$ of the last plasma dilution exhibiting each function (i.e., a dilution of 1:8 is scored as 3). Lysis reflects the interaction of NAbs and lytic enzymes (e.g., complement); agglutination results only from NAb activity.

To the protocol described by Matson et al. (2005), I made two minor modifications. First, I used plates processed for improved hydrophilic qualities (Corning Costar #3798, instead of #3795). Second, for all steps requiring phosphate buffered saline (PBS), I used Dulbecco's PBS (#D8662; Sigma; St Louis, MO). Because this formulation includes $MgCl_2*6H_2O$ and $CaCl_2*2H_2O$ in addition to the basic ingredients, its use counteracts any effects of plasma serial dilution on endogenous divalent cation concentration. The sources of the exongenous RBCs were farm-raised rabbits; whole blood was collected on heparin as a byproduct for purposes of biological research (#RBA050; HemoStat Laboratories; Dixon, CA).

Haptoglobin Quantification

Haptoglobin (Hp) is an acute phase protein found in a wide range of taxa including birds (Delers et al. 1988). I followed the "manual method" instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc.; Morris Plains, NJ) to quantify the concentration (mg/mL) of Hp in all plasma samples. This colorimetric assay is based on the binding properties

of Hp and haemoglobin and the peroxidase activity of free haemoglobin. I performed the assay at room temperature in 96-well microtiter plates using 7.5 µL of plasma. Absorbance was recorded at 630 nm five minutes after reaction initiation using a microplate reader (VERSAmax; Molecular Devices; Sunnyvale, CA). I serially diluted the calibrator (provided at a known concentration of 2.0 mg/mL) with diluent to generate a standard curve for use in calculating concentrations from absorbance values. A positive control (pooled plasma samples from house sparrows, *Passer domesticus*) was run in duplicate in every plate, and Hp concentrations were standardized among plates based on the mean with-plate positive control value.

Blood Smear Evaluation

A single blood smear from each individual was evaluated by conducting differential counts and estimating the overall leukocyte concentration (Feldman et al. 2000). Differentials were determined by counting individual cell types until a cumulative total of 100 leukocytes was reached. Total leukocyte concentrations were estimated by averaging the number of leukocytes in ten microscope fields at high power and multiplying this mean value by 2000 to approximate the number per uL (Bounous & Stedman 2000). From these data, concentrations (#/µL) of heterophils, lymphocytes, monocytes, eosinophils, and basophils were estimated (Bounous & Stedman 2000). All blood smears were evaluated blind to species by a single veterinary diagnostic laboratory technician (AVL Veterinary Clinical Laboratory; St Louis, MO).

References

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