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Quantifying and comparing constitutive immunity across avian species

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Abstract

Studies that blend a comparative approach to immunology with an appreciation for physiological ecology are defining an important new field in biology—ecological immunology. However, a panel of assays that permits a comparative approach to immunology is not yet available. In this paper, we describe several assays of innate immunity that do not require species-specific reagents and therefore ideal for use in comparative immunology studies. We optimized the assays for use in small birds, where sample volumes are limiting. The bactericidal assay measures the capacity of whole blood to kill microorganisms, and integrates many important components of constitutive immunity. The phagocytosis assay measures the phagocytic capacity of macrophages in whole blood. Bioassays for mannan binding protein and lysozyme can be used to measure inflammation-induced levels of these acute phase proteins in the plasma. Species differences in bactericidal and phagocytic activities against *Staphylococcus aureus* and *Escherichia coli* were observed in populations of captive and in free-living birds, demonstrating the assays' utility for multi-species comparisons. However, clay-colored thrushes (*Turdus grayi*) that were stressed by prolonged capture and handling had diminished phagocytic and antibacterial activities, indicating the need to conduct these assays soon after capture. When birds were challenged with lipopolysaccharide (LPS), levels of mannan-binding protein, lysozyme, and haptoglobin were elevated and bactericidal and phagocytic activities of blood were altered, indicating that these measurements are sensitive to the current infection status of the animal. All assays could be done on as little as 10 μL of blood or plasma and should be useful in field studies of comparative immunity.
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1. Introduction

At its most fundamental level, comparative immunology involves assessment of immune processes of a wide variety of species. Comparisons between closely related species provide an understanding of the fine tunings of the immune system to specific environments while comparisons across orders, families, and genera permit discovery of

Abbreviations: LPS, lipopolysaccharide; MBP, mannan-binding protein, PAMP, pathogen-associated molecular pattern

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diverging immunological strategies. A complete evaluation of immunity requires assessment of both the innate and adaptive components of immunity.

Studies that blend a comparative approach to immunology with an appreciation for physiological ecology and evolution are defining an important new field in biology—ecological immunology [1–5]. The development and action of different immune system components require resources such as energy and nutrients during different life stages of an animal. Because the costs of different immune system components differ, a bias favoring some types of defense over others is hypothesized to reflect trade-offs between competing life-history traits [5–7]. Unfortunately, a panel of assays that permits a comparative approach to immunology is not yet available. Conventional techniques for assessing immune function utilize species-specific reagents and are usually not suitable for use across species. For example, several human interleukins, interferons, and chemokines have greatly diminished or no bioactivity in birds [8]. Similarly, antibodies produced against CD molecules, regulatory peptides, or effector molecules of one species often do not recognize those of another species, or their binding affinities are sufficiently different to compromise quantitative analysis. Finally, the culture of leukocytes is typically done using media fortified with serum or purified serum components that are species-specific, including complement and other pathogen-recognizing factors [9,10], growth factors, nutrient profiles, and nutrient binding proteins like transferrin [11–13].

In addition to the challenge of developing assays that are not species-specific, several other factors complicate comparative studies. First, stress markedly changes many indices of immune function and non-domestic animals, especially free-living individuals, are highly susceptible to the stress of capture and handling. Furthermore, animals may show different physiological responses to stress depending on species, gender, season, and many other factors, leading to differing impacts on immune function [14]. Thus, the study of captured individuals may introduce artifacts due to different responses to the stress of captivity or the husbandry conditions. Secondly, terminal sampling is often undesirable or prohibited, so assessments of immune function of many species are best accomplished using blood or benign biopsies. Finally, many species of interest are small in size and sample volume is a limiting factor. Assuming blood contributes 6% of body weight and that it is acceptable to remove 10% of the blood volume, only about 60 μL of blood is available for evaluation of immune function in a 10 g bird. In summary, a panel of assays useful for comparisons across species is most useful if reagents are species-independent, if it does not require holding animals in captivity, if it uses small blood samples, and if the results are quantitative and integrate across important cellular and regulatory processes that predict immune function. Such a panel would have the most utility if time-sensitive steps can be accomplished in field laboratories with limited infrastructure.

Given these considerable limitations, innate immunity lends itself to comparative study better than adaptive immunity. The innate immune system consists of both constitutive and inducible components. The constitutive component includes epithelial integrity, various antimicrobial peptides and proteins, and the antimicrobial functions of heterophils, macrophages, and other phagocytes. The inducible component of innate immunity includes the systemic acute phase response, which is highly effective but a costly defense in terms of energy expenditure and behavioral changes [15].

Our approach was to develop and validate assays that use small sample volumes of whole blood or plasma from newly captured birds. We have previously described an assay to quantify natural antibodies and complement-like lytic activity using small samples of blood plasma [16]. Here, we describe two assays that examine constitutive innate components of immunity of whole blood, bacterial killing, and phagocytosis. Furthermore, we optimized an assay for two antimicrobial plasma proteins, lysozyme, and mannan-binding protein (MBP) for use with small birds. These assays were developed using blood and plasma samples from captive birds and analyzed in a conventional immunology laboratory, then their utility was tested on samples from wild birds in a minimally equipped field laboratory.

2. Materials and methods

2.1. Animals and bleeding

Chickens (*Gallus gallus*, Hyline single comb white leghorns for experiments on bursectomy and Cobb × Cobb broilers for all other experiments), American kestrels (*Falco sparverius*), cockatiels (*Nymphicus hollandicus*), and zebra finches (*Taeniopygia guttata*)

were housed at the University of California, Davis Avian Biology research facility. Clay-colored thrushes (*Turdus grayi*), blue-gray tanagers (*Thraupis episcopus*), crimson-backed tanagers (*Ramphocelus dimidiatus*), variable seedeaters (*Sporophila americana*), and white-tipped doves (*Leptotila verreauxi*) were captured near the Smithsonian Tropical Research Institute in Gamboa, Panama (9°12″N, 79°42″W). Birds were captured using mist nets, and a sterile blood sample was taken within 3 min of capture because the stress hormone corticosterone has been shown to be at or near baseline levels in birds for 3 min following capture [17].

To provide a sterile bleeding site, feathers were removed from the area surrounding the brachial vein and the site was swabbed liberally with 70% ethanol in order to mat down remaining feathers and remove dander. After air-drying (approximately 20 s), the site was swabbed with a fresh ethanol-saturated cotton ball. It was found to be important to let the ethanol completely evaporate before taking the sample because the drying process is important for sterilization and because ethanol causes hemolysis, which can complicate some assays. After ethanol had completely evaporated, a vein was punctured with a 26 gauge needle and blood was collected directly into sterile heparinized capillary tubes (50 μL capacity) before it had a chance to escape the sterilized area. A clay card was sterilized with ethanol and used to plug the tube. Prior to plugging, the capillary tube was held horizontal to permit the formation of a small air bubble at the end of the tube. This bubble separated the blood sample from the clay, preventing contamination of the sample with fine particles that might be phagocytosed. The card and tube were then placed in a covered plastic container that had been sterilized with ethanol and then transported to the lab. Protecting the process from wind-blown dust and insuring that the bleeder did not breathe on the bleeding site were also found to be important for sterility. Husbandry practices and blood collection protocols were approved by the UC Davis Institutional Animal Care and Use Committee.

2.2. Assays

2.2.1. Microbicidal assay

The method of Keusch et al. [18] for measuring bactericidal activity of leucocytes in whole blood was modified and adapted for smaller sample sizes. *Candida albicans* (American Type Culture Collection (ATCC) # 10231), *Escherichia coli* (ATCC#8739 or 51813) and *Staphylococcus aureus* (ATCC#6538) supplied as 10^7 or 10^8 organisms per lyophilized pellet (Epower Assayed Microorganism Preparation; Microbiologics Inc., Saint Cloud, MN) were reconstituted according the manufacturers instructions in 40 mL of sterile, endotoxin-free PBS (tissue culture grade). *E. coli* strains 11775 and 23716 were obtained directly from ATCC (Manassas, VA 20108). Reconstituted pellets were kept for up to one week at 4 °C in sterile PBS and very little bacterial growth was observed. Each day that the assay was run, the stock culture was diluted with cold PBS to produce a working culture with 50,000 bacteria/mL. This working solution was kept on ice at all times.

Whole blood was diluted to the indicated amounts with pre-warmed (41 C) CO_2-independent media (#18045; Gibco-Invitrogen, CA, California) plus 4 mM L-glutamine in a sterile 1.5 mL capped tube. Ten microliters of the bacterial working culture (about 200 bacteria) was added per 100 μL of diluted blood, vortexed, and incubated for the indicated time at 40.5 °C. The samples were removed from the incubator, vortexed, and duplicate 50 or 75 μL aliquots were pipetted onto agar plates, spread, inverted, and incubated at room temperature. Colonies were counted 24 h (for bacteria) or 48 h (for yeast) later. The number of bacteria in the initial inoculum was determined by diluting bacteria in media alone (10 μL of the working culture per 100 μL of media) and then immediately plating. All pipetting and plating procedures were done inside a laminar flow hood (field lab: Airclean 600, Airclean Systems, Raleigh North Carolina; UCDavis lab: Sterile Guard, The Baker Company, Sanford, ME). Sterility controls in which no bacteria were added were also employed for experiments conducted in the field. The antimicrobial activity of blood was defined as the percent of the inoculum killed, which was calculated as 1—(viable bacteria after incubation/number inoculated).

2.2.2. Phagocytosis assay

The assay of Sandgren et al. [19] for measuring the phagocytic activity of isolated neutrophils was adapted for measuring the phagocytosis activity of adherent cells in whole blood. *E. coli* (E-2864) and *S. aureus* (S-2854) bioparticles® labeled with the fluorescent tag BODIPY® FL (Molecular Probes,

Eugene, OR) were reconstituted with tissue-culture-grade PBS plus 2 mM sodium azide according to the manufacturers instructions. Bacteria suspensions were diluted to the appropriate concentration with CO_2-independent media (Gibco-Invitrogen) supplemented with 1% pen/strep and 4 mM L-glutamine and pre-warmed to 40.5 °C before use. Blood was diluted 1:20 with CO_2-independent medium containing glutamine and antibiotics and 66 μL was pipetted into eight-well chamber slides (Nalge Nunc International, Naperville IL). Two hundred and fifty microliters of diluted bacteria were added to each well and the chamber slides were incubated for the indicated periods of time at 40.5 °C. After the incubation, phagocytosis was stopped by cooling the slide on ice. The medium, which contained the erythrocytes, heterophils, and thrombocytes, was removed from the adherent cells by washing the slide gently twice with 300 μL of cold media. Based on morphology, the adherent cells were predominantly macrophages. The slide was then fixed by adding 300 μL of 100% methanol for 5 min on ice. The methanol and chamber housing were removed and the slide was stored in a light-proof container until evaluation.

At a later date, the slides were examined using a fluorescent microscope with an excitation/absorption spectrum of 505/513 at 40 × magnification. Phagocytosis was scored by determining the percentage of macrophages containing at least one fluorescent particle. At least 100 cells were counted per chamber section.

2.2.3. Acute phase proteins

To measure plasma lysozyme levels, the lyso-plate assay of Osserman and Lawlor [20] was adapted for use with 10 μL plasma samples in 96-well plates and for obtaining a quantitative endpoint. This assay measures the lysis of bacteria as indicated by a decrease in opacity of an agar–bacteria suspension. Briefly, 25 mg dried *Micrococcus lysodeikticus* (M3770-5G; Sigma-Aldrich, St. Louis, MO) was added to 50 mL sterilized 1% agarose (0140-01, Difco laboratories, Detroit, Mi, USA) and kept at a temperature of 50–60 °C. In a 96-well plate, 150 μL (unless otherwise specified) of this suspension was added to a 10-μL plasma sample. A standard curve was obtained by adding the bacterial suspension to serial dilutions (10 to 0.3125 mg/L) of a standard chicken lysozyme solution (Sigma-Aldrich). Absorbance was measured in a Versamax microplate reader (Molecular Devices, Sunnyvale CA, USA). The optimum absorbance value was determined (see results) to be 850 nm and this value was used for subsequent experiments.

MBP concentrations were determined by an ELISA. In this assay, plasma MBP was immobilized onto plastic and its binding capacity was saturated with FITC-conjugated mannan, which was quantified using anti-FITC-Ig conjugated to horseradish peroxidase. Briefly, 145 μL of carbonate buffer, pH 9.6 (C-3041; Sigma-Aldridge), was added to each well of a flat bottom polystyrene 96-well microplate followed by 5 μL of either plasma or standard MBP solution. A serial dilution (5–0.039 mg/mL) of human MBP (C4121478; US Biological, Swampscott, Massachusetts) served as a standard. Plates were incubated overnight at 4 °C and then washed twice with 250 μL of washing buffer (PBS + 0.05% Tween20, P-3563; Sigma-Aldrich). Blocking solution (4% BSA treated with 10 mM sodium periodate for 6 h, then dialyzed against PBS overnight) was added and plates were incubated overnight at 4 °C. Wells were then washed twice with buffer and 150 μL of mannan reagent (0.15 mM Mannan FITC conjugate in PBS with 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$) was added. After incubating overnight at room temperature, wells were washed twice and 150 μL of anti-FITC antibody (1:3000 anti-FITC-HRP, AB045P; Chemicon Inc, Temecula, CA in blocking buffer) was added. Plates were incubated for 2 h at room temperature, washed twice, and 100 μL of substrate (BM Blue POD Substrate, # 11484281001, Roche Inc, Nutley, NJ) was added. After 15 min, the reaction was stopped with 50 μL of 1 N H_2SO_4 and absorbance was determined at 450 nm.

Haptoglobin concentrations were determined using a commercial kit (Tri-Delta Diagnostics Inc., Morris Plains, NJ) that measures the capacity of haptoglobin to bind heme and preserve its peroxidase activity at a low pH.

2.3. Optimization experiments

2.3.1. Bacterial killing assay

The bactericidal assay was tested over a range of incubation times, blood dilutions, and bacterial concentrations to optimize these parameters. The effect of diluting heparinized whole blood on the viability of leukocytes over time was determined in order to define the range of blood dilutions that could be considered in developing assays utilizing whole blood. The effect of incubation time and of

dilution on the antimicrobial activity of whole blood from chickens and cockatiels was tested against *S. aureus*, *C. albicans*, and four different strains of *E. coli*. In these experiments, blood was taken from three chickens (4 weeks of age) and three adult cockatiels.

2.3.2. Phagocytosis assay

A series of experiments were conducted to optimize incubation time, blood dilution, bacteria concentrations, pre-assay blood storage time, number of washes, and fixation method. To determine the role of complement, an experiment was conducted where blood cells were separated from plasma by centrifugation and complement was deactivated at 57 °C for 30 min and then added back to the cells from which they were separated.

2.3.3. Lysozyme

A series of experiments were performed to determine the optimal amount of agar to be added to 96-well plates, the incubation time before reading, the change in the spectral pattern of the wells during incubation, and the linear working range of measurement.

2.4. Validation experiments

2.4.1. Bactericidal and phagocytic activities in captive and free-living birds

To determine if species differences could be detected with the new assays, bactericidal and phagocytic activities against *E. coli* and *S. aureus* were measured in four species of captive birds (Davis, California) and five species of free-living birds (Gamboa, Panama). The species tested were selected as a matter of convenience and not based on phylogenetic criteria.

2.4.2. Effect of capture stress on bactericidal and phagocytic activities

To determine the effect of the stress of capture and handling on the assays of immune function, four clay-colored thrushes were bled immediately after capture (time 0) and then placed in a cloth bag. At 30 and 60 min later, they were bled again. Blood samples were assayed for phagocytosis activity in March of 2004 and for bactericidal activity in March of 2005.

2.4.3. Sensitivity of antimicrobial and phagocytic activity to an acute phase response

During an infectious challenge, innate immunity is bolstered by neutrophilia and by some of the acute phase proteins. Thus, an experiment was conducted to determine if the microbicidal activity of blood was sensitive to the acute phase response caused by lipopolysaccharide (LPS; *Salmonella typhimurium* prepared by trichloroacetic acid extraction, Sigma-Aldrich). Two-week-old chicks were administered one of three treatments: no injection, LPS-injection or antibiotic injection (positive control). The LPS was injected subcutaneously (1 mg/kg body weight) and blood was sampled 16 h later. The chickens in the antibiotic group were injected with 1 mg/kg colistin (colistin sodium methanesulfonate, Fluka, Switzerland), which is commercially known as polymyxin E and has bactericidal activity against Gram-negative bacteria. Blood was sampled 1 h later. The 48 chicks were housed at three chickens per pen and one chick in each pen was randomly assigned to each treatment. Bactericidal activity towards *E. coli* (ATCC # 51813) and *C. albicans* (ATCC # 10231) was examined on whole blood samples from 48 chicks and phagocytosis of *E. coli* was determined in whole blood from 24 chicks. At the time of blood collection for immune assays, additional plasma was collected and frozen for subsequent determination of acute phase proteins.

2.4.4. Role of specific IgY in bactericidal and phagocytic activities

Chickens were chemically bursectomized using cyclophosphamide as described by Glick and Olah [21]. Blood from 14-day-old control and bursectomized chickens ($n = 5$) was assayed for phagocytosis of and bacterial killing activity. The extent of the bursectomy was examined by weighing the remaining bursa. All bursectomized chicks had bursal weights that were less than 20% of control chicks.

2.5. Corticosterone measurement

Corticosterone in plasma from chickens was measured using a commercial kit (Immunodiagnostic Systems, Fountain Hill, AZ). Corticosterone in plasma from free-living birds was measured by radioimmunoassay [22]. Antibody was purchased from Esoterix Endocrinology (Calabasas Hills, CA) and radioactive label from NEN Life Science Products (Boston, MA). We added 20 μL of trace

label to determine the percent recovery (mean ± SE, 74.3 ± 0.6%) after extraction of the plasma samples with dichloromethane. Samples that were below the lower detection limit of the assay (1.25 ng/mL) were set at detection limit for a conservative estimate. All samples were run in a single assay.

2.6. Statistics

Effects of species or treatment (dilution, time of incubation, number of bacteria, bursectomy, colistin, or LPS, depending on the experiment) were analyzed by one-way analysis of variance. When the main effect was significant, Least Significant Difference or Student's *t*-Stests were used to compare the group means. All assays were conducted in duplicate or triplicate but the bird was considered as the experimental unit, and treatment as a fixed variable. Time series (stress test on clay-colored thrushes) were analyzed using repeated measure analysis, with treatment as fixed effect and bird as the experimental unit.

3. Results

3.1. Optimization experiments

3.1.1. Bactericidal assay

Whole blood dilutions of 1:4 or more resulted in better viability of blood leukocytes than lower dilutions and viability did not decline at high dilutions (Table 1); consequently all experiments used a dilution of 1:4 or greater. The effect of dilution of chicken blood on the microbicidal activity of whole blood against *S. aureus*, or four different strains of *E. coli* is shown in Fig. 1a. Diluted whole blood was bactericidal for all strains. There was wide variation in the susceptibility of the three strains of *E. coli* to the bactericidal actions of blood, with ATCC # 51813 being least susceptible and # 8739 being most susceptible. In general, dilution of whole blood decreased the bactericidal activity towards *E. coli* similarly across strains. *S. aureus* was more resistant to the bactericidal effects of whole blood than most of the *E. coli* strains and

Table 1
Effect of dilution of whole chicken blood on viability (%) of white blood cells[a]

Dilution	Time of incubation		
	24 h	48 h	72 h
1:0	74	49	44
1:1	79	73	52
1:2	94	94	88
1:4	96	95	97
1:10	97	97	96
1:20	98	93	95
1:50	92	94	93

[a]Whole blood from chickens ($n = 3$) was diluted with medium (vol:vol) and 100 μL was incubated at 41 °C in 96-well plates. Viability was determined by exclusion of trypan blue.

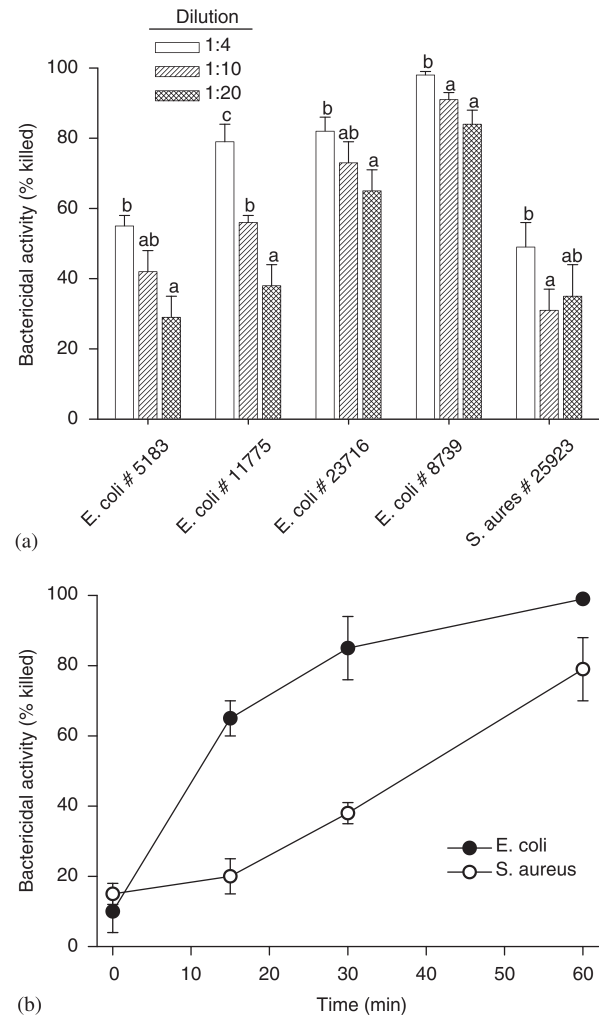

Fig. 1. (a) Effect of dilution of whole blood from chickens on the bactericidal activity against *E. coli* (ATCC # 8739; 5183; 11775; or 23716) and *S. aureus* (ATCC # 25923). Blood was diluted (vol:vol) either 1:4, 1:10, or 1:20 with medium and incubated with bacteria for 30 min. Means not sharing common superscripts are significantly different ($P < 0.05$). (b) Effect of time of incubation on the bactericidal activity of whole blood from chickens. Blood was diluted 1:10 and incubated with *E. coli* (ATCC # 8739) or *S. aureus* (ATCC # 25923) for the indicated times. For both experiments, data are mean + SEM; $n = 3$.

the dilution response was not linear. As expected, the time of incubation markedly affected the bactericidal activity of dilute chicken blood (Fig. 1b). In the case of *S. aureus*, the effect of time was linear from 15 to 60 min ($r^2 = 0.84$; $P < 0.03$). For *E. coli* # 8739, most of the bactericidal action was expressed in the first 30 min.

3.1.2. Phagocytosis assay

In preliminary experiments, it was found that monocytes in whole chicken blood adhered to the chamber slide within 15 min, so this was used as the minimum incubation time in subsequent experiments. In preliminary work, it was also found that washing twice at the end of the incubation step removed sufficient numbers of RBCs and unattached white blood cells to permit accurate scoring for phagocytosis. Of the dilutions tested (1:4, 1:10, and 1:20), 1:20 was optimal for recovery of monocytes and lower dilutions resulted in clumping of cells and loss of yield (data not shown). The number of bacteria added to whole blood influenced the proportion of adherent cells that internalized bacteria (Fig. 2a). *S. aureus* at a 1:200 leukocyte to bacteria ratio and *E. coli* at a 1:100 ratio resulted in levels of phagocytosis that were about half of maximal, so this concentration was used in subsequent studies. The proportion of adherent cells that internalized bacteria increased linearly with the incubation time (Fig. 2b).

Storage of blood on ice for 1 h prior to adding bacteria and incubation at 40.5 °C resulted in a marked decrease in phagocytosis activity (Fig. 2c). Storage at room temperature did not result in a significant decrease ($P = 0.04$ versus time 0) in phagocytic activity until 2 h of storage. However, storage for 1 h resulted in a decreasing trend ($P = 0.14$) in *S. aureus* phagocytosis. For this reason, all subsequent assays were conducted within 30 min of blood collection, during which time blood was stored at ambient temperature.

3.1.3. Lysozyme

In a first experiment, different amounts of agar and bacteria suspension (20, 40, 60, 80, 100, 150, and 300 μL) were added to 10 μL of a serial dilution of lysozyme standard. While volumes of agar–bacteria suspension above 40 μL resulted in good linear correlations between absorbance and lysozyme concentration, adding 150 μL gave the most consistent results as indicated by the greatest linearity of standards between 5 and 0.625 mg/L after 16 h incubation ($R^2 = 0.98$ for the average of eight dilution series). Consequently, 150 μL of agar–bacteria suspension per well was used in further experiments.

A scan of the absorbance spectra of agar alone or the agar–bacteria mix did not reveal any peaks in

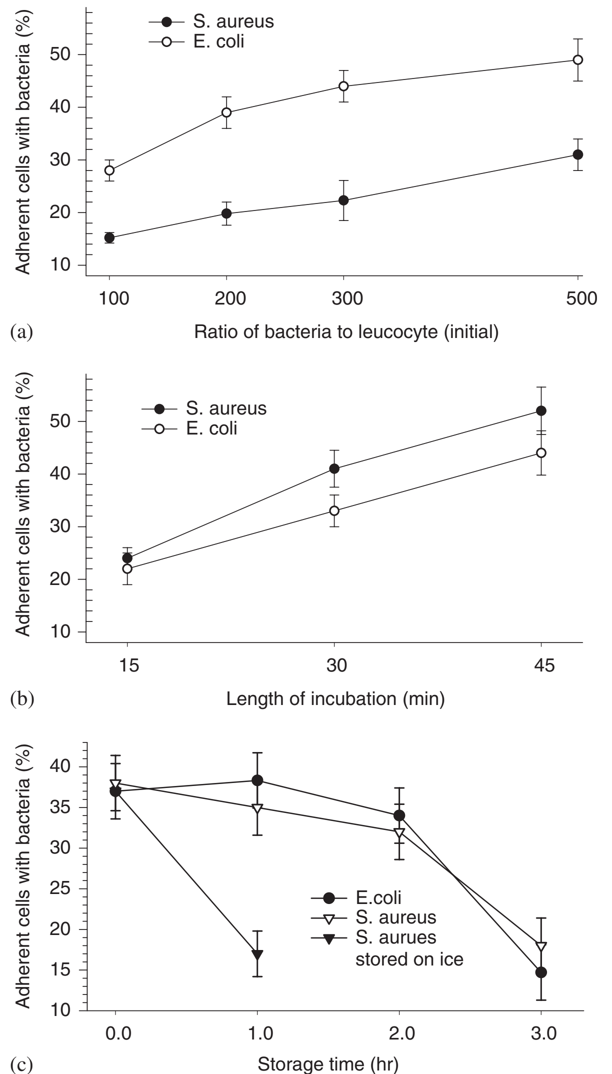

Fig. 2. (a) Influence of the bacteria:leucocyte ratio on the number of adherent cells with internalized fluorescently labeled bacteria (*E. coli* E-2864 and *S. aureus* S-2854). Bacteria were added to give a ratio of bacteria to adherent cells of 100:1, 200:1, 300:1, or 500:1 and incubated for 15 min. (b) Influence of the time of incubation on the number of adherent cells with internalized fluorescently labeled bacteria. Bacteria were added in a 100:1 (*E. coli*) or 200:1 (*S. aureus*) bacteria:leucocyte ratio and incubated for 15, 30, or 45 min. (c) Influence of storage conditions of blood prior to assay. Blood was stored at room temperature or on ice for the indicated periods of time and then tested for the phagocytic capacity of adherent cells. Bacteria were added in a 100:1 (*E. coli*) or 200:1 (*S. aureus*) bacteria:leucocyte ratio and incubated for 30 min. For all experiments, data are mean + SEM; $n = 3$ chickens.

absorbance between 300 and 950 nm. Although reading serial dilutions of the lysozyme standards at different wavelengths between 340 and 850 nm gave different standard curves (i.e. different *y*-intercepts), their correlation coefficients were not affected by wavelength. In further experiments, we measured at 850 nm because this wavelength is relatively insensitive to hemoglobin, which could contaminate some plasma samples.

The optimal time for reading the absorbance of the plates was determined using serial dilutions of lysozyme from 10 to 0.625 mg/L. With time, the decline in absorbance plateaued and this nadir was dependent upon the concentration of the standard. Thus, it was not possible to measure a concentration of 5 mg/L at a time that also optimized reading low concentrations. Adding more bacteria to the agar (0.75, 1, or 1.25 mg/mL instead of 0.5 mg/mL) did not affect the kinetics of agar clearing. An incubation period of 16–20 h was acceptable for most physiological samples and also resulted in a standard curve with the greatest slope and highest correlation coefficient. Samples containing high levels of lysozyme (e.g. those from infected birds) are best evaluated after 1 h of incubation using a separate standard curve.

3.2. Validation experiments

3.2.1. Bactericidal and phagocytic activities in captive birds and free-living birds

The bactericidal activity of diluted blood differed among captive species raised at the same animal facility (Table 2). Diluted blood from Zebra finches was significantly less capable of killing *S. aureus* than that of other species. Blood from American kestrels was better at killing *E. coli* than blood from chickens and tended to be better than that of cockatiels ($P<0.08$). Cockatiel monocytes were less capable of phagocytosis of either *S. aureus* or *E. coli* compared to chicken or American kestrel monocytes.

The bactericidal activity of diluted blood differed across free-living species in Panama living in the same habitat (Table 3). Blood from clay-colored thrushes and crimson-backed tanagers killed significantly more *E. coli* and *S. aureus* than did blood from white-tipped doves or blue-gray tanagers. Variable seedeaters were relatively proficient at killing *E. coli*, but not *S. aureus*. Monocytes from blue-gray tanagers phagocytosed the most *E. coli* and cells from white-tipped doves phagocytosed the least. Monocytes from white-tipped doves were similarly poor at phagocytosing *S. aureus*. The ability of diluted blood of the five species to kill *E. coli* was significantly correlated ($P = 0.02$; $R^2 = 0.67$) to their ability to kill *S. aureus*.

3.2.2. Effects of baseline and stress-induced corticosterone levels on bactericidal and phagocytic activities

Clay-colored thrushes that were stressed by capture and handling increased their circulating concentrations of corticosterone ($P<0.01$), and decreased the capacity of their monocytes to phagocytose *S. aureus* at 30 and 60 min ($P = 0.05$), and tended ($P = 0.08$) to decrease phagocytosis of *E. coli* (Fig. 3a). In a second experiment using a larger number of birds, bactericidal activity against *E. coli* was significantly ($P = 0.04$) but weakly negatively correlated with corticosterone levels at the time of capture ($r^2 = 0.195$). Corticosterone levels were significantly increased after 30 min stress ($P<0.01$), but there

Table 2
Comparison of the bactericidal and phagocytic activity of diluted whole blood from different species of captive birds

Species	Bactericidal activity (% killed)		Phagocytosis (%)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Chicken	79 ± 7^{b}(4)	$68^{a}\pm4$(5)	$22^{b}\pm3$(3)	$26^{b}\pm4$(3)
Zebra finch	45 ± 6^{a}(2)	not done	$18^{ab}\pm2$(3)	$16^{ab}\pm2$(3)
Cockatiel	76 ± 3^{b}(5)	$81^{ab}\pm5$(3)	$10^{a}\pm2$(3)	$8^{a}\pm2$(3)
American Kestrel	78 ± 2^{b}(4)	$95^{b}\pm2$(6)	$22^{b}\pm2$(4)	$29^{b}\pm3$(4)

Bactericidal assay: blood was diluted 1:10 and incubated with *E. coli* (ATCC #51813) or *S. aureus* (ATCC #25923) for 30 min. Phagocytosis assay: blood was diluted 1:20 and incubated with *S. aureus* or *E. coli* for 30 min. Data are presented as mean + SEM (*n*). Means not sharing common superscripts are significantly different ($P<0.05$).

Table 3
Comparison of the bactericidal and phagocytic activity of diluted whole blood from different species of wild Panamanian birds

Species	Bacteriocidal activity		Phagocytic activity	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
Blue-gray tanager	28 ± 3^{a}	22 ± 3^{ab}	52 ± 6^{c}	22 ± 3^{ab}
Crimson-backed tanager	75 ± 6^{b}	31 ± 3^{b}	37 ± 3^{b}	27 ± 3^{b}
Clay-colored thrush	85 ± 5^{b}	29 ± 4^{b}	41 ± 5^{bc}	25 ± 4^{b}
Variable seedeater	72 ± 7^{b}	19 ± 5^{ab}	39 ± 5^{bc}	14 ± 4^{a}
White-tipped dove	30 ± 4^{a}	11 ± 3^{a}	14 ± 3^{a}	15 ± 3^{a}

Assay conditions were the same as described in Table 2. Means not sharing common superscripts are significantly different ($P < 0.05$).

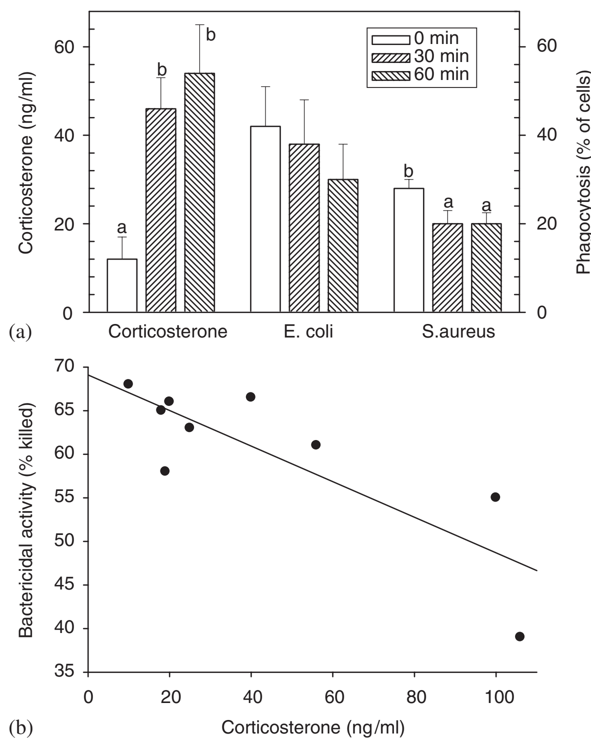

Fig. 3. (a) Plasma corticosterone concentrations and capacity of blood adherent cells to phagocytose *E. coli* in clay-colored thrushes immediately at capture (0 min) and after 30 or 60 min of capture and handling stress (mean + SEM; $n = 4$). Means not sharing common superscripts are significantly different ($P < 0.05$). (b) Correlation between plasma corticosterone concentration and bactericidal activity against *E. coli* 8739 of diluted blood from clay-colored thrushes ($n = 4$) immediately after 30 min of capture stress.

was considerable individual variation in the amount of this increase (Fig. 3b). Bactericidal activity was diminished with increasing corticosterone levels and this correlation was highly significant ($R^2 = 0.606$, $P < 0.01$, Fig. 3b).

3.2.3. Sensitivity of antimicrobial and phagocytic activities to an acute phase response

The bactericidal activity of diluted chicken blood against *E. coli* was increased by antibiotic treatment ($P = 0.02$) and tended to be increased ($P = 0.08$) following an acute phase response to LPS (Fig. 4a). The antimicrobial activity against *C. albicans* was significantly ($P = 0.04$) diminished following an acute phase response to LPS injection (Fig. 4b). As expected, antibiotic treatment did not impact the ability of diluted blood to kill of *C. albicans*. Phagocytosis of *E. coli* was increased following an acute phase response to LPS ($P < 0.01$, Fig. 4c). LPS challenge significantly ($P < 0.05$) increased the concentrations of plasma lysozyme (6.5 ± 2.0 μg/mL in controls versus 13.5 ± 2.6 μg/mL in LPS challenged birds; mean + SEM), haptoglobin (1.2 ± 0.2 mg/mL in controls versus 1.9 ± 0.2 mg/mL in LPS challenged birds) and MBP (4.9 ± 2.8 μg/mL in controls versus 11.2 ± 3.6 μg/mL in LPS challenged birds) 16 h after injection.

3.2.4. Role of specific IgY in bactericidal and phagocytic activities

Bursectomy had only a minor effect on the bactericidal and phagocytic activity of whole blood from chicks (Table 4). Phagocytosis of *S. aureus* and killing of *E. coli* strain 5183 and *C. albicans* was not significantly affected by bursectomy. Phagocytosis of *E. coli* and killing of *E. coli* strain 8739 were decreased by 31% and 26% by bursectomy, respectively.

4. Discussion

The vertebrate immune system consists of many interacting components, each with specific protective value and inherent metabolic costs of production

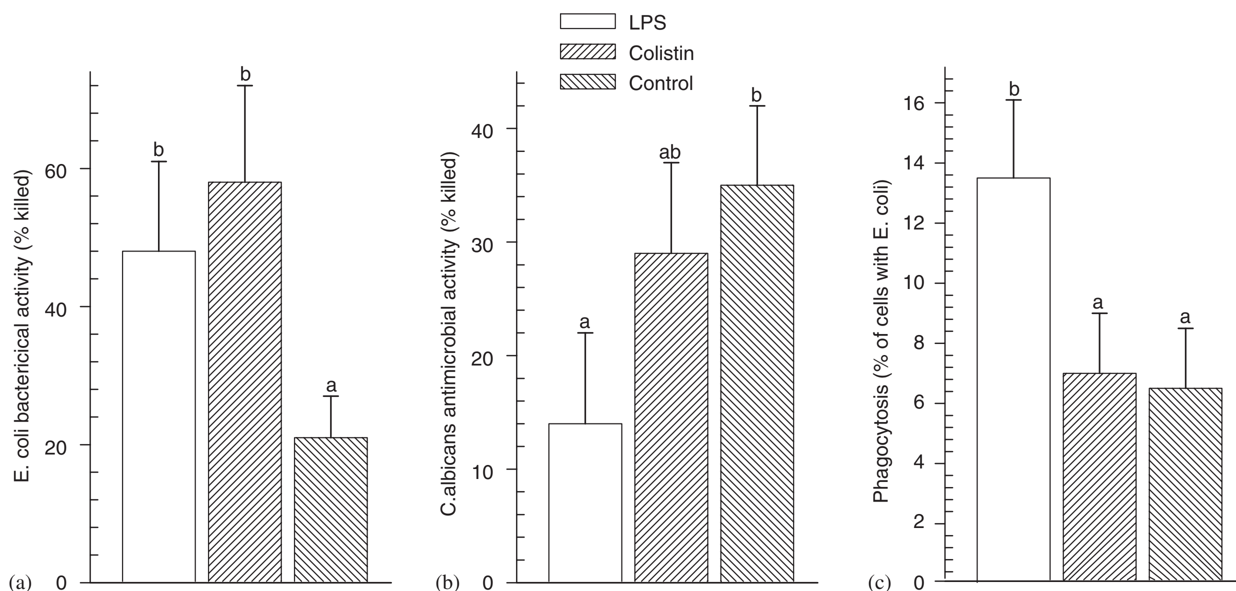

Fig. 4. (a) Effect of LPS or colistin (polymyxin E) injection on the bactericidal activity against *E. coli* (ATCC # 51813 of whole blood from chicks (n = average of three pens). (b) Effect of LPS or colistin injection on the antimicrobial activity against *C. albicans* (ATCC # 10231) of whole blood from chicks (n = average of three pens). (c) Effect of LPS or colistin injection on the phagocytic capacity of chicken adherent cells (monocytes; n = average of three pens). Results are expressed as the percentage of monocytes that internalized fluorescently labeled *E. coli* (E-2864).

Table 4
Effect of bursectomy on the phagocytosis and killing of microorganisms by chicken blood

Parameter	Control	Bursectomized
Phagocytosis of *E. coli* (%)	29.7±2.5	20.6±2.4*
Phagocytosis of *S. aureus* (%)	22.6±1.7	19.9±1.5
Killing of *E. coli* 5183 (%)	42.3±4.3	44.4±4.0
Killing of *E. coli* 8739 (%)	88.7±8.2	65.2±5.1*
Killing of *S. aureus* (%)	29±2.8	26±1.7
Killing of *C. albicans* (%)	36.2±4.0	33.8±4.8

Phagocytosis was determined by adding bacteria at 100:1 (*E. coli* E-2864) or 200:1 (*S. aureus* S-2854) bacteria:leucocyte ratio and incubated for 30 min. Data are expressed as % of adherent cells that internalized fluorescent-labeled bacteria. To measure microbicidal activity, blood was diluted 1:10 and incubated with bacteria for 30 min or with *C. albicans* for 1 h. The number of viable organisms at the end of the incubation period is expressed as % of the number of the initial inoculum. Mean±SEM (n = 4).
*Significantly different from control ($P<0.05$).

and use. The nascent field of ecological immunology endeavors to understand the diversities in immune systems of animals and relate these differences to variation in ecological and evolutionary factors within and between species [5–7,23]. Unfortunately, progress in developing tools for making interspecies comparisons of immune defenses has been limited. The microbicidal, phagocytosis, and acute phase protein assays described in this study, together with measures of natural antibodies and complement [16], should be useful for gauging the constitutive innate immunity of birds and other taxa.

For the bactericidal and phagocytosis assays, our approach was to develop and validate assays that use whole blood instead of purified leukocytes. Assays based on whole blood are advantageous because they allow very small sample volumes, better reflect the in vivo state, and minimize artifacts that result from removal of the nutritional and regulatory factors found in plasma and replacing them with fetal calf serum. We were able to conduct most of these assays on 10 μL blood samples from 15 g zebra finches and therefore the methods we describe could in theory be used to safely assess birds of any size.

The bactericidal assay gives an index of the capacity of the blood to rapidly thwart a potential pathogen. This activity is probably a combination of several mechanisms of the innate immune system, including phagocytic activities of leukocytes and microbicidal activities of humoral proteins such as natural and specific antibodies, lysozyme, and opsonins. Thus, it is probably the most general

and integrative in vitro measurement of the constituent elements of the innate immune system.

The strains used were chosen because they are not known to be pathogens for humans or animals and can be handled safely using biosafety level 1 protocols. Most of these strains are available from commercial suppliers in standardized pellets that make their use in a field lab possible. Furthermore, their genera are commonly abundant in nature and are commensal in vertebrates. Because animals will have coevolved with these or very similar strains, they likely have constitutive components of their immune systems that are effective in limiting their viability. We preferred the use of these test species in contrast to rarer or more pathogenic microorganisms because of the risk that some, but probably not all, birds would have been previously exposed and have specific immunity. The presence of specific antibodies would bias the results, add to the variability of the assay, and compromise the interpretation of the results. We found that the elimination of specific antibody by embryonic bursectomy reduced the ability of diluted blood to kill one out of the four microbes tested and it reduced phagocytosis of one of the two bacteria tested. This indicates that most of the chicks used in this study had specific antibody that participated in the killing or phagocytosis of some of the test strains. For applications of these assays to free living populations, the use of many test strains should minimize the confounding effect of specific antibody on the interpretation of the results. However, confirmation of the lack of specific antibody (e.g. by western blots) is the best way to clarify interpretation.

There were large differences in the ability of blood to kill different microbes. *E. coli* 8739 was most susceptible to killing while *E. coli* 5183, *S. aureus*, and *C. albicans* were least susceptible. We found that almost all of the bactericidal activity of blood against *E. coli* 8739 resided in plasma and that cellular components contributed little. Conversely, the cellular components were required for effective killing of the other strains (data not shown). Thus, assays using each test strain are likely probing different components of the immune system and multiple strains will likely be needed to provide a general index of microbicidal capacity. We found that the ability of the five Panamanian species to kill *E. coli* was correlated ($r^2 = 0.67$) with their ability to kill *S. aureus* and we are currently examining such correlations in a much greater number of species using a greater diversity of microbes. Given that the correlation between the killing of two types of bacteria is only modest, the use of several test strains is more likely to provide a robust evaluation.

Interspecies differences in microbicidal activity can be further probed by examining the ability of blood phagocytes to internalize fluorescently labeled bacteria. The short incubation period used with our assay (30 min) followed by removal of non-adherent cells resulted in the enrichment of blood monocytes and the loss of the other major phagocytic cells, namely thrombocytes and heterophils. As with the microbicidal assay, phagocytic capacity could be determined on very small blood samples (10 μL) and retains the plasma components during the assay. Regardless of species, phagocytosis of *S. aureus* was lower than that of *E. coli*. In addition to the assay of adherent cells (macrophages), we found it possible to remove the non-adherent cells at the end of the incubation, fix them with 2% formaldehyde, and evaluate phagocytosis by granulocytes and thrombocytes. However, the amount of phagocytosis by non-adherent phagocytes was low and the high number of contaminating erythrocytes made evaluation very labor intensive.

The antimicrobial and phagocytic activities of blood were dependent on the time between collection of blood and its analysis. For reliable comparisons, either between or within species, it is important that the protocol is highly standardized. In the field, there is likely to be a lag time between the collection of samples and their delivery to a field lab. This lag should be kept less than 1 h and our data suggests that less than 30 min is ideal. Storing samples on ice markedly diminished the phagocytosis activity of the blood and samples should not be cooled prior to assay. Presumably this is because cooling decreases the metabolic activity and phagocytic capacity of phagocytes. The optimal length of incubation during the assay was considered to be that which results in 50% killing and was dependent on the strain.

There was a clear effect of capture and handling stress on bactericidal activity. Corticosterone is a primary, though not the only, mediator of stress influence on the immune system [24]. There was a good correlation between corticosterone levels in birds stressed for 30 min and their bactericidal activity. It is likely that the diminution in bacterial killing due to stress is partially due to effects on phagocytes (Fig. 3). For blood sampled immediately

after capture, a negative correlation between corticosterone levels and bactericidal activity was significant but was not robust ($r^2 = 0.195$). Because birds were bled in less than 3 min after they contacted the nets, the variation in initial corticosteroid levels is likely to be due to time of year, sex, or stresses experienced in the wild and not due to capture stress [17,25].

The microbicidal and phagocytosis assays were developed using parameters (time, dilution, etc.) that are appropriate for chickens. We confirmed these parameters in cockatiels (data not shown), but not in other species. When used for interspecific comparisons, these parameters should be useful as a first screen for species differences. Once important species differences are found, investigators should confirm that the differences are not an artifact of species-dependent assay parameters such as dilution, incubation time, or incubation temperature.

Colistin, also called polymyxin E, consists of polypeptides with bactericidal activity against Gram-negative bacteria [26–28], so it was used to test our ability to measure an increase in blood bactericidal activity. In this experiment, colistin increased *E. coli* killing but was not completely bactericidal. Although an intravenous dose of colistin in chickens gives a peak concentration after 1 h that is effective in vivo [29], it is likely that the 10-fold dilution of plasma with antibiotic-free media resulted in a suboptimal concentration of colistin in our experiment. Nevertheless, colistin was useful as a positive control for validating that an increase in bactericidal activity of blood could be measured.

LPS induced an acute phase response as indicated by elevated levels of MBP, lysozyme, and haptoglobin. An acute phase response elicited by LPS increased the bactericidal activity of the blood to a similar extent as colistin. LPS from the cell wall of Gram-negative bacteria triggers an innate immune response by inducing the production of pro-inflammatory cytokines that stimulate acute phase protein production [30–32]. Several of the acute phase proteins opsonize bacteria and enhance phagocytosis. We observed enhanced phagocytosis of *E. coli* 16 h after LPS and this likely contributed to the observed increase in bactericidal activity.

We were surprised that *C. albicans* killing was impaired during the acute phase response because the acute phase protein serum amyloid A enhances anti-*Candida* activity of phagocytes [33] and impairment of phagocytosis is a pathogenic mechanism in fungal infections [34]. However, Kitz et al. [35] showed that rabbit MBP inhibited phagocytosis of *C. albicans* by murine macrophages and MBP was elevated in our chickens.

Our assays for acute phase proteins should be useful for interspecies comparisons because they measure bioactivities of the proteins and do not rely on species-specific reagents. Lysozyme, discovered by Fleming [36] in the first part of the 20th century, exerts antibacterial properties by hydrolyzing β-1,4-linkages between *N*-acetylmuraminic acid and *N*-acetyl-D-glucosamine residues in the peptidoglycan of bacterial cell walls [37]. The lysozyme assay evaluates this activity by measuring the lyses of *M. lysodeikticus*. We modified the method of Osserman and Lawlor [20] to accommodate very small samples and for the use of a microplate reader to measure the bacteria lysis in agar. MBP is a serum collectin that opsonizes a wide variety of microorganisms and activates complement. The MBP assay takes advantage of the lectin activities of this protein. MBP was elevated following LPS injection. MBP has been shown to be increased during viral infections in chickens [38,39]. Like the assay for lysozyme, the assay for MBP is possible on plasma sample as small as 10 μL; however, the MBP assay is more variable and more time consuming than the lysozyme and haptoglobin assays. Additionally, it is possible that at least some of the mannan-binding activity that is being detected is due to anti-mannan antibodies. Thus, this assay is likely to be appropriate for examining changes in MBP following an acute phase response, but the absence of anti-MPB antibodies should be confirmed when measuring constitutive levels of MBP. The assay for haptoglobin measures heme-binding activity in the plasma and, as such, likely measures PIT54 in chickens [40]. Lysozyme, MBP, and haptoglobin were constitutively present in the plasma of healthy chicks and their measurement in wild birds might be used as an index of their current level of infection or, if healthy, their investment in constitutive innate immunity.

The tests described here cover several aspects of the constitutive innate immune system. While the bactericidal assay is a general measure of constitutive innate immunity, the phagocytosis assay focuses on an important component of cellular innate immunity and acute phase proteins are an index of the humoral component of the innate

immune system. Matson et al. [16] reported species-non-specific assays that measure natural antibody and complement-like activities in small plasma samples. Together, the combination of these assays should provide insight in the constitutive immunity of wild birds. However, an accurate assessment of the immune system will require measuring both constitutive and inducible immune defenses.

Measurement of acute phase proteins following an LPS injection may be used to evaluate induced innate immunity. Injecting birds with LPS increased bactericidal activity, phagocytosis, and acute phase protein concentrations. Responses to LPS and other PAMPs are evolutionarily conserved across vertebrates and invertebrates and are fundamental to the response to most infectious challenges [3]. Three defenses against pathogens are greatly enhanced by PAMPs [41]: (1) opsonization of bacteria and viruses for phagocytosis or activation of the lectin pathways of complement, (2) uptake of pathogens by phagocytes and dendritic cells, and (3) triggering of signaling pathways that result in the induction of transcription of immune response genes for anti-microbial peptides and inflammatory cytokines. Our assays allow the evaluation of each of these defenses. However, as mentioned above, investigators should be careful in drawing conclusions from experiments that include the holding of wild birds in captivity because of the confounding effects of stress.

5. Conclusions

Assays were optimized to measure constitutive innate immunity on small blood volumes using species-non-specific reagents. These tests will be a useful tool to evaluate innate immunity in a wide variety of species. The observed variability in measures of innate immunity between free-living species from the same habitat suggests that it could be a useful tool to understand the ecological and evolutionary divergence in immune strategies that might underlie the diversification of life-history strategies [23]. The more difficult task of evaluating adaptive immunity mediated by lymphocytes will need to be solved before a complete index of the immune defenses of free-living animals can be made.

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