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Commissioned Article

Does antibody binding to diverse antigens predict future infection?

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SUMMARY

We studied diverse antigen binding in hosts and the outcome of parasitism. We used captive-bred F1 descendants of feral rock pigeons (Columba livia) challenged with blood-feeding flies (Hippoboscidae) and a protozoan parasite (Haemoproteus). Enzyme-linked immunosorbent assays (ELISAs) and immunoblots were used to test (i) whether pre-infection IgY antigen binding predicts parasite fitness and (ii) whether antigen binding changes after infection. Assays used extracts from three pigeon parasites (northern fowl mite, Salmonella bacteria and avian pox virus), as well as nonparasitic molecules from cattle, chicken and keyhole limpet. Binding to hippoboscid and S. enterica extracts were predictive of hippoboscid fly fitness. Binding to extracts from hippoboscids, pox virus and nonparasitic organisms was predictive of Haemoproteus infection levels. Antigen binding to all extracts increased after parasite challenge, despite the fact that birds were only exposed to flies and Haemoproteus. Immunoblots suggested innate Ig binding to parasite-associated molecular markers and revealed that new antigens were bound in extracts after infection. These data suggest that host antibody binding to diverse antigens predicts parasite fitness even when the antigens are not related to the infecting parasite. We discuss the implications of these data for the study of host–parasite immunological interaction.

Keywords *apicomplexan, avian, ELISA, immunoglobulin, innate immunity, parasite*

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INTRODUCTION

Identifying predictors of susceptibility vs. resistance among hosts is vital for understanding the dynamics of host–parasite interaction and mitigating infectious disease. Predictors of infection are wide ranging and include genetic, physiological, behavioural and ecological factors (1, 2). Among immunological factors, antibodies (immunoglobulin; Ig) are often used as diagnostic indicators of host–parasite interaction, because they bind molecules (antigens) associated with parasite identity and function. Antigen binding enables the immune system to recognize parasites and mobilize defensive proteins (e.g. complement) and cells (e.g. phagocytes) that engage and eliminate the infection (3). In addition, antibodies may directly harm a parasite by binding to receptors necessary for parasite fitness (reproductive success), as occurs with virus neutralization. The various roles antibodies play in host resistance to parasites underscore the importance of these humoral defences to the outcome of host–parasite interaction.

Antibodies are produced in both the innate and adaptive compartments of the immune system (3–6). Natural antibodies are produced primarily by B-1 lymphocytes and are present in the immune system without the stimulus of infection (4–6). These antibodies are believed to (i) provide a first line of defence against infection and (ii) facilitate the adaptive immune response after infection (7, 8). In contrast, antibodies produced through the adaptive immune response are made by B-2 lymphocytes stimulated by antigens introduced during infection (3, 4). Among all antibodies, the specificity of antigen binding is variable. Monoreactive antibodies are highly specific and bind to a single antigen, or closely related antigens. In contrast, polyreactive antibodies (pAbs) are able to bind a wide range of potentially unrelated antigens (9). The natural antibodies are predominantly polyreactive, whereas adaptive antibodies are believed to be primarily monoreactive (10). Regardless of antigen specificity, or the category of immune response (innate vs. adaptive), antibody-mediated

defences originate with antibody binding to an antigen that has not been encountered. As such, the capacity of the host's antibodies to bind antigens before infection may influence the host–parasite interactions that follow.

Several lines of evidence indicate that the antibody repertoire prior to parasite exposure will influence the outcome of infection. Innate antibodies have been isolated that bind to parasite-associated molecular patterns (PAMPs), facilitating parasite recognition and elimination. For example, mice (*Mus musculus*) produce immunoglobulin M (IgM) that will bind to phosphocholine – a constituent of the cell wall of *Streptococcus pneumoniae* – without prior exposure to the bacteria (i.e. natural IgM) (11). Injection of those antibodies into naive mice reduces the mortality caused by subsequent infection with *S. pneumoniae*, illustrating a protective effect of innate Ig binding to PAMPs (12). Uninfected hosts also possess innate polyreactive antibodies that bind to both self-antigens and viral antigens. For example, polyreactive IgM, IgG and IgA have been isolated from humans that will bind to human IgG (i.e. self-reactive antibodies) and are protective against HIV infection *in vitro*, implicating a role for pAbs in protection against viruses (13). Importantly, pAbs can be stimulated by infections, which suggests that such antibodies are not exclusive to the innate humoral immune response (5, 14).

In field studies of wild host–parasite systems, researchers have measured antigen binding using the hemagglutination-haemolysis assay (HHL). The HHL assay measures *in vitro* antibody binding to vertebrate red blood cells, which are not expected to contain antigens associated with parasites, or antigens encountered naturally in the host environment (15). Thus, the assay reflects the capacity of circulating Ig to bind to diverse molecules. The levels of antibodies detected in this assay have been inversely correlated with parasite infection in wild populations. For example, Whiteman *et al.* (16) observed that Galapagos hawks with high levels of antibody binding to rabbit red blood cells had low ectoparasite loads, suggesting that diverse antigen binding provided protection against blood-feeding arthropods.

These studies imply that antibodies expressed before infection can influence host–parasite interactions. However, properties of the antibodies that coordinate these effects remain unclear, because laboratory and field studies have measured different things. Laboratory-based experiments with model species have focused on isolated innate antibodies that bind to specific PAMPs, whereas field studies on wild species have used assays that detect broad antigen binding. Two overriding questions remain: (i) Are host–parasite interactions predicted by antibody binding to molecules from any potential parasite, or only select parasites? (ii) Are antigens unassociated with parasites also predictive of host–parasite interaction? These questions are relevant to practical concerns in studies of wild populations. With wild animals, it is difficult to determine and isolate relevant antigens from the diverse community of parasites that may have infected hosts. In addition, species that do not have particular parasites may not respond to selected PAMPs from those organisms, limiting comparative studies of immune function.

In contrast, if antigens that are unassociated with parasites are effective indicators of host–parasite interaction, then standard antigens (e.g. keyhole limpet haemocyanin) may be used in field and comparative studies with greater confidence. The two overriding questions stated above are also relevant to conceptual issues in parasite immunology. For example, evolutionary studies have explored the role of parasites in shaping immune function and immunogenetic variation. This is exemplified by studies of the major histocompatibility complex (MHC), where it is hypothesized that diversity of the parasite community influences the diversity of the MHC alleles present in the host population (17, 18). As with the MHC receptors, antibodies play a role in self- vs. non-self recognition (5, 19). The diversity of antigens that are bound by innate antibodies could hypothetically respond to selection pressures imposed by parasites. Exploring such co-adaptive interactions requires a clear understanding of the relationships between antibodies, PAMPs and antigen diversity.

One approach to these questions is to utilize model organisms where B lymphocytes, Ig molecules and antigens could be easily isolated and characterized. This approach maximizes control over molecular details, but potentially lacks relevance to diverse natural systems. An alternative approach is to use a natural host–parasite system where parasite exposure can be controlled and antigenic diversity is based on taxonomic and ecological properties that are meaningful to the system. This approach provides limited molecular insights, but is informative of broad patterns. In this study, we have taken the latter approach. We tested the hypothesis that binding of circulating antibodies to previously un-experienced antigens will predict the outcome of parasite infection in a natural host–parasite system. Using serum collected from the captive-bred F1s of wild-caught rock pigeons (*Columba livia*), we measured IgY binding to proteins derived from parasitic and nonparasitic organisms with enzyme-linked immunosorbent assays (ELISAs) and Western blots. The assay proteins were extracted from species that the host birds had not encountered before the study. These represented the taxonomic groups Arthropoda, Bacteria, Mollusca, Vertebrata and DNA viruses. Serum samples were taken before and after the host birds were exposed to two different parasites: a blood-feeding fly

(*Pseudolynchia canariensis*) and a blood parasite that the fly transmits, *Haemoproteus columbae*. Fly reproduction and replication of *H. columbae* were recorded post-challenge to determine the fitness of each type of parasite. Using these measures, we addressed the following specific questions: (i) Does total IgY predict parasite reproductive fitness after challenge? (ii) Does IgY binding to proteins from novel sources predict parasite reproductive fitness after challenge? (iii) Does either total IgY, or the antigen-binding profile change after parasite exposure? Our central goal was to better understand the role of antigen binding in host–parasite interaction. We discuss our results in the context of host–parasite immunology and disease ecology.

MATERIALS AND METHODS

This experiment used samples from a recently reported study of pigeon (*C. livia*) defences and infection with a blood parasite (*H. columbae*) transmitted by the blood-feeding fly *P. canariensis* (20). Serum samples collected before and after parasite exposure were screened in separate assays to measure total IgY in circulation and the binding of IgY to different proteins. Measures of IgY were then compared to the measures of parasite reproduction observed on the host birds (Figure 1).

Animal husbandry, infection and sample collection

The study used pigeons that were bred in captivity from feral parents. Additional details on pigeon husbandry practices in this study are described elsewhere (21). All birds were at least 6 months of age at the time of the experiment, and reproductively mature; thus, their innate immune systems were expected to reflect adult levels (22). Briefly, experimental birds ($N = 23$) were split into two groups. Half of the birds were fitted with bits, which are harmless c-shaped pieces of plastic that fit into the nares of the pigeon and prevent them from grabbing/damaging flies with the beak (21). The other half was not bitted (normal beak function). These two groups of birds were evaluated to isolate immune-mediated effects on parasitism (birds with bits) from the combination of behavioural and immune-mediated effects (birds without bits).

Flies were reared from a source colony and subsequently infected with *H. columbae* by feeding the flies on culture birds with confirmed *H. columbae* infections. The flies were then transferred to enclosures containing the experimental birds. Individual birds were maintained with the flies for a 5-week period, which is more than enough time for birds to establish *H. columbae* infections. Parasite fitness (fly reproductive output) was measured by (i) counting the number of pupae female flies produced

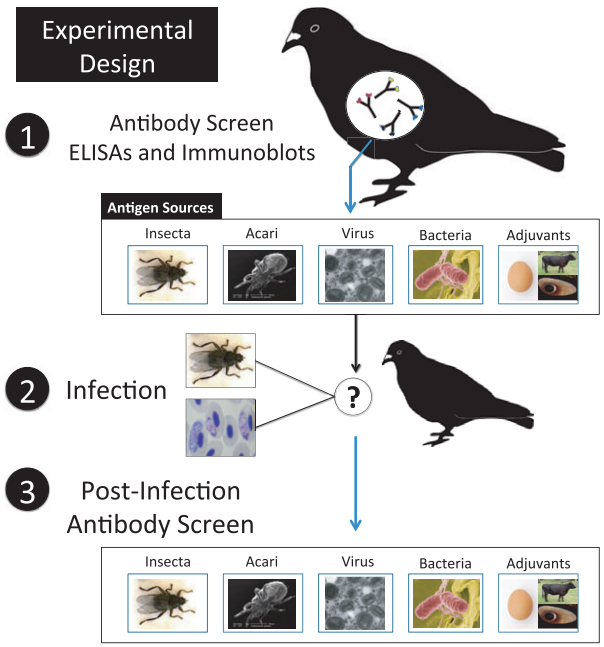

Figure 1 Experimental approach. We took serum samples from pigeons and screened the samples for antibodies binding to extracts from insects (*P. canariensis*), mites (*O. sylviarum*), viruses (avipox), bacteria (*S. enterica*), chickens (*G. gallus*), cattle (*B. taurus*) and mollusks (*Megathura crenulata*). The captive bred pigeons used were not previously in contact with any of these organisms. We asked two overriding questions (A & B): (A) Does the binding of antibodies to these extracts (un-experienced antigens) (Step 1) predict the outcome of subsequent infections with blood feeding flies (*Pseudolynchia*) and malaria (*Haemoproteus*) (Step 2)? (B) Does parasite infection (Step 2) affect the antigen binding profile (Step 3)? This experimental design allowed us to test the following prediction: Diversity of antigen binding prior to parasite infection predicts parasite fitness upon infection.

weekly, as described in (20), and (ii) estimating intensities of the blood parasite *H. columbae* from thin blood smears made every 3 days from the time of exposure until the end of the experiment. Maximum infection intensity was used as an estimate of *H. columbae* population level fitness.

Focal serum samples were collected from pigeons 1 week prior to being infested with hippoboscid flies (see below), and again 5 weeks after fly exposure. Experiments were performed in accordance with IACUC guidelines at the University of Utah (Protocols #08-08004 and #11-07018).

Methods for comparing total IgY

Total IgY was compared among birds using a standard sandwich ELISA based on the protocol provided by Bethyl Laboratories for chicken IgY quantification (see supporting information for the detailed protocol). Briefly, 96-well microtiter plates (Costar™ Corning, Tewksbury, MA, USA)

were coated with antigen extracts and incubated overnight at 4°C. Plates were washed with Tris buffer and then incubated 30 min with a blocking solution (bovine serum albumin). Blocking solution was removed (3× wash), serum was added (1 : 100 dilution in Tris + BSA sample buffer), and the plates were incubated for 1 h on an orbital shaker at room temperature. Serum samples were then removed (3× wash), and a detection antibody was added (goat anti-bird IgY, h+l chain – HRP conjugated; Bethyl Labs, Montgomery, TX (USA)). The detection antibody was diluted 1 : 50 000 in sample buffer. Plates were incubated 1 h on an orbital shaker at room temperature. Detection antibody was removed (3× wash), and the HRP substrate was added (TMB). The colour reaction was stopped after 15 min with addition of 0·18 M H_2SO_4. Colour intensity (optical density; OD) was measured at 450 nm using a microplate reader (Bio-Rad iMark Bio-Rad, Hercules, CA (USA)). Triplicate OD values for each bird were averaged for use in statistical analyses.

Methods for measuring novel antigen-binding IgY

Five sandwich ELISAs were used to measure IgY binding to proteins from (i) *P. canariensis* (Hippoboscid), (ii) *Ornithonyssus sylviarum* (northern fowl mite), (iii) *Salmonella enterica* bacteria, (iv) fowl pox virus (Aviviridae) and (v) a protein mixture consisting of bovine serum albumin (BSA) + chicken ovalbumin (OVA) + keyhole limpet haemocyanin (KLH). Importantly, the pigeons never encountered these organisms during the experiment, with the exception of the hippoboscid flies, which were only encountered during the controlled infestations (i.e. after the prechallenge serum was collected). The ELISAs were developed following the methods described in (14), (see supporting information for complete protocols). The ELISA for binding to *S. enterica* used bacteria (*Abony serovar*) from lyophilized preparations (Microbiologics, St. Cloud, MN (USA)™) suspended in PBS. The ELISA for binding to fowl pox virus used a vaccine (Pfizer, New York, NY (USA)™) preparation of live virus in PBS. The ELISA for binding to adjuvant proteins used commercial preparations of mariculture keyhole limpet hemocyanin (Pierce Imject™ mcKLH), ovalbumin (Pierce Imject™ OVA) and bovine serum albumin (Pierce Biotechnology, Thermo Scientific, Rockford, IL (USA)™ BSA) in equal concentrations (1 mg/mL) suspended in MES buffer (0·05 M 2-[*N*-morpholino]-ethanesulfonic acid, pH 4·7). Based on measured protein concentrations or reported concentrations of commercial preparations, the assays were based on the following amounts of material: fly and mite extracts (~1·8 μg/well), bacteria (~5000 colony forming units/well), pox virus (~1 dose/well) and adjuvant (3 μg/well).

Western blots of assay antigens

Antigen preparations were separated using polyacrylamide gel electrophoresis (PAGE). We combined 40 μL of each protein solution with 35 μL of Laemmli sample buffer (Bio-Rad) and 5 μL 2-mercaptoethanol. Samples were incubated at 70°C for 10 min and then loaded onto a 10–20% Tris-HCl polyacrylamide gel (Bio-Rad Criterion™ Precast Gel). Electrophoresis was run in tris-glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0·1% SDS, pH 8·6) at 200 V for 1 h. A gel was stained with Coomassie (Bio-Rad, Hercules, CA (USA)™) and imaged using a Bio-Rad imaging system. Separate, matching gels were used to transfer the separated extracts to PVDF membranes using the Bio-Rad Trans Blot Turbo system. Post-blotting, membranes were submerged in blocking solution (3 g nonfat milk powder in 60 mL TTBS (20mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20 (pH 7.5))) and incubated overnight at 4°C. Each membrane was then incubated overnight at 4°C with pooled serum from either pre-infection samples, or from post-infection samples (500 μL serum diluted in 100 mL TTBS). The membranes were then washed five times with TTBS and then incubated with 3% goat serum for 60 min at room temperature. Blots were then incubated with detection antibody (goat anti-bird IgY heavy + light chain – HRP) diluted 1 : 50 000 in TTBS for 60 min at room temperature. Finally, blots were covered with chemiluminescent reagent (Bio-Rad Clarity™ substrate) and imaged (Bio-Rad Chemi Doc™).

Statistical analyses

Measures of total IgY and antigen binding were tested for correlations, and OD values were compared between pre- and post-challenge serum samples using a paired *t*-test (SYSTAT 12 Systat Software, San Jose, CA (USA)). Generalized linear models were used to test the effects of pre-challenge antibody measures on (i) the total number of fly pupae and (ii) the maximum count of *Haemoproteus*-infected cells observed for each bird using a mixed general linear model (SYSTAT 12). The model included bit treatment, pre-challenge ELISA measures and interactions for each pre-challenge ELISA × bit treatment as fixed effects. Bird was treated as a random factor. Counts of fly pupae were normally distributed and were analysed without transformation. Counts of *Haemoproteus*-infected cells, which were not normally distributed, were square root-transformed. The transformed data met assumptions of normality. Given that data were analysed relative to two response variables, we applied a Bonferroni correction to the significance level and report significance at $P \leq 0{\cdot}025$.

Factors with probability values between 0·025 and 0·1 are reported as trends. From the results of each ELISA, we determined the upper quartile cut-off value among the birds in the bitted and nonbitted treatment groups. Using these cut-off values for each assay, we categorized each serum sample (bird) as *high binding* if the optical density value was in the upper quartile for the respective treatment group. For each bird, we counted the number of high-binding ELISA results observed out of the five antigen assays (hippoboscid, fowl mite, pox virus, *S. enterica* and adjuvants). These counts were used as relative measures of antigen-binding diversity, such that more assays in the high-binding category reflected greater diversity of antigen binding.

Antibody binding patterns in Western blots were compared with published immunoblots of hosts exposed to the same parasites, or related taxa. The fluorescence intensities of bands were measured using Quantity One software v.4.1 (Bio-Rad). The intensities of bands observed on both pre- and post-infection blots were compared after standardizing to the reference ladder included in blots.

RESULTS

Measures of total IgY and antigen binding were uncorrelated (Table 1). Correlations of pre- vs. post-challenge antigen binding OD values were weak among the birds, with total IgY measures showing the strongest relationship ($R = 0{\cdot}84$). Overall, there was no indication of an identity effect, whereby some birds had consistently high or low binding in pre- vs. post-challenge samples. Total IgY did not differ between pre- and post-challenge samples across all birds (paired $t = 0{\cdot}55$, df = 21, $P = 0{\cdot}591$). In contrast, all measures of antigen binding increased post-challenge (paired $t > 3{\cdot}47$, df = 21, $P < 0{\cdot}005$) (Figure 2).

The IgY quantity and binding to extracts varied in their predictive power of fly or blood parasite reproductive success (Figure 2). Fly pupae counts were inversely related to total IgY ($t = -3{\cdot}60$, $P = 0{\cdot}011$). There was also an inverse trend with IgY binding to adjuvant proteins ($t = -2{\cdot}00$, $P = 0{\cdot}09$). There were significant interactions between bit treatment and total IgY ($t = 3{\cdot}99$, $P = 0{\cdot}007$), binding to hippoboscid extracts ($t = -3{\cdot}91$, $P = 0{\cdot}008$) and binding to *S. enterica* extracts ($t = 3{\cdot}13$, $P = 0{\cdot}020$). Birds ($n = 7$) that had high binding (upper quartile of OD values) in three or more ELISAs had total pupae counts at or below the median number of pupae in each bit treatment (Figure 3). Birds with fewer than three high-binding ELISA results ($n = 16$ birds) had total pupae counts that ranged from the minimum to the maximum observed counts.

Maximum blood parasite count was positively related to IgY binding with adjuvant proteins ($t = 3{\cdot}51$, $P = 0{\cdot}013$) and inversely related to the interaction of bit treatment and adjuvant binding ($t = -4{\cdot}35$, $P = 0{\cdot}005$) (Figure 2). These opposing relationships were driven by a disparity in OD values between the bitted and nonbitted groups. No high-binding values were observed with adjuvant proteins among samples from the nonbitted treatment group. A negative trend was observed with binding to hippoboscid extract and infected cell counts ($t = -2{\cdot}31$, $P = 0{\cdot}060$). Binding to pox extract showed a positive trend alone ($t = 2{\cdot}34$, $P = 0{\cdot}058$) and a negative trend interacting with bit treatment ($t = -2{\cdot}67$, $P = 0{\cdot}037$). These differing relationships had opposing slopes in the two bit treatment groups. Birds that had high binding in three or more ELISAs had infected cell counts near or below the median

Table 1 Pairwise correlation coefficients for OD values from ELISAs. Shaded boxes indicate strong (dark grey) and moderate (light grey) correlations, ignoring corrections for multiple comparisons. After Bonferroni correction for multiple comparisons, none of the correlations was statistically significant

	Pre-Total IgY	Pre-Hipp	Pre-Mite	Pre-Pox	Pre-Sal	Pre-Adjv	Post-Total IgY	Post-Hipp	Post-Mite	Post-Pox	Post-Sal	Post-Adjv
Pre-Total IgY	1·000											
Pre-Hipp	0·223	1·000										
Pre-Mite	0·017	0·588	1·000									
Pre-Pox	0·163	0·107	0·239	1·000								
Pre-Sal	0·219	0·208	0·050	0·063	1·000							
Pre-Adjv	0·436	0·459	0·356	0·100	0·316	1·000						
Post-Total IgY	0·835	−0·004	0·319	0·050	0·038	0·100	1·000					
Post-Hipp	0·291	0·230	0·335	−0·162	−0·086	0·310	0·446	1·000				
Post-Mite	0·083	−0·073	0·158	−0·225	−0·016	−0·126	0·410	0·453	1·000			
Post-Pox	0·104	0·063	0·467	−0·017	−0·211	−0·113	0·378	0·371	0·640	1·000		
Post-Sal	0·011	−0·099	−0·201	0·063	−0·250	−0·076	0·162	0·262	0·576	0·659	1·000	
Post-Adjv	0·353	−0·616	−0·305	−0·279	0·188	0·002	0·492	0·129	0·698	−0·224	0·546	1·000

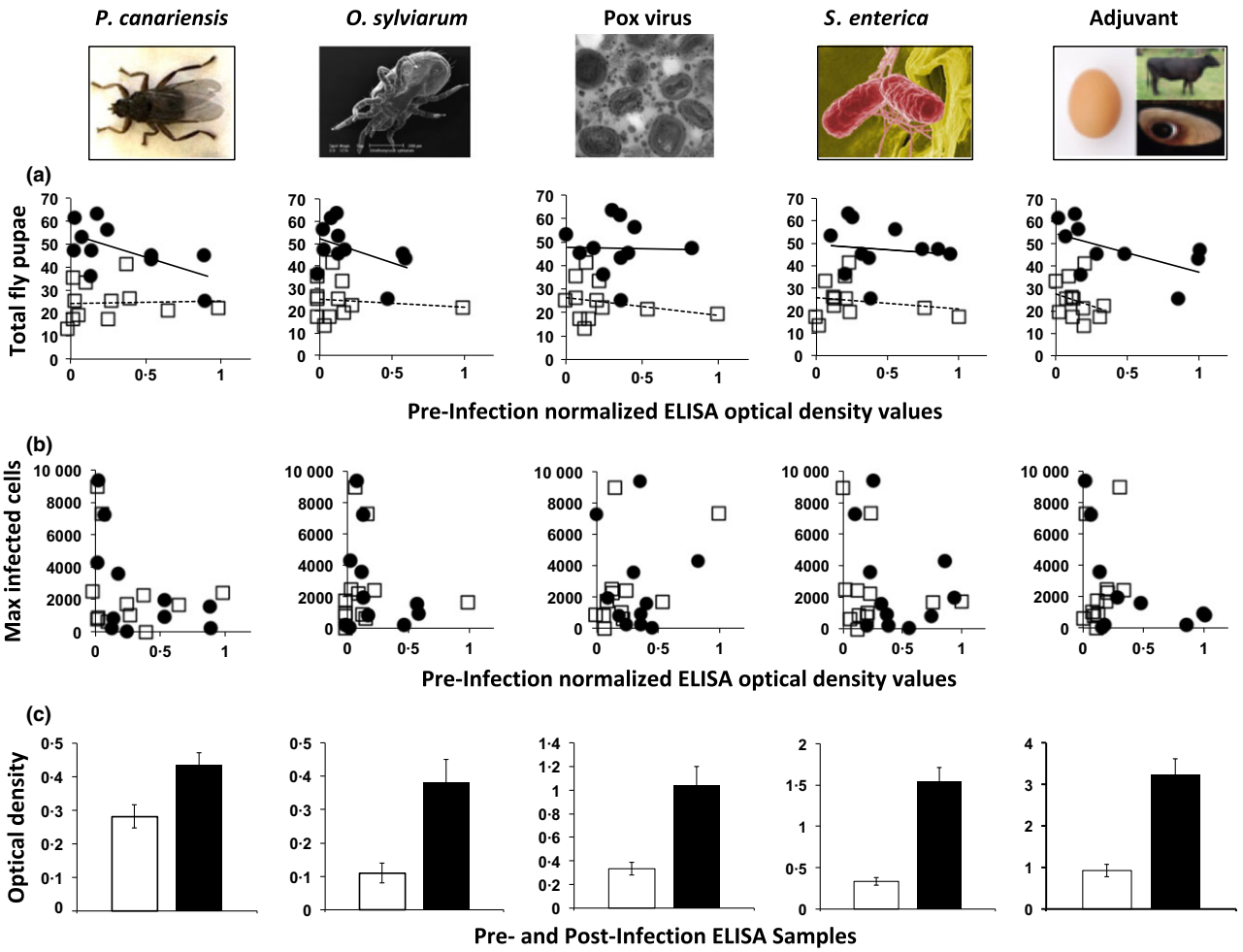

Figure 2 Antigen binding and parasite measures. Photographs along the top illustrate the sources of antigens screened in ELISAs with serum samples taken before and after birds were challenged with hippoboscid flies (*P. canariensis*) and avian malaria (*H. columbae*). The panels in each column represent data for the extracts from the source(s) depicted at the top. (a) The total count of *P. canariensis* pupae (fly reproduction; *y*-axis) is shown relative to ELISA values (antigen binding; *x*-axis). Pigeons were either bitted to impair preening of flies (circle, solid fit line), or not bitted (square, dotted fit line). (b) The maximum count of malaria-infected blood cells (malaria reproduction; *y*-axis) is shown relative to ELISA values (antigen binding; *x*-axis). (c) Average (±SE) ELISA values (*y*-axis) are shown for serum samples collected before parasite exposure (open bar) and 5 weeks after parasite challenge (closed bar).

(Figure 3). In contrast, birds with fewer than three high-binding ELISA results had infected cell counts that ranged from the minimum to the maximum counts.

The Western blots confirmed antigen binding with pre- and post-infection serum samples for all extracts (Figure 4). With the exception of *S. enterica,* all bands detected with pre-infection serum were also detected with post-infection serum. The intensities of bands observed with both serum samples did not differ for any extract (paired $t = 2{\cdot}36$, $P = 0{\cdot}59$). This suggests that antibodies binding to those specific antigens did not become more numerous after infection. For all extracts, there were new bands observed after infection, which indicated that the range of antibody–antigen binding increased after exposure to parasites. For extracts of hippoboscid flies, multiple new bands appeared with post-infection serum, which likely reflected adaptive antibodies produced in response to the flies. Fewer new bands appeared post-infection for the other extracts, which were derived from organisms not involved with the infections.

For extracts from parasitic organisms (hippoboscid, pox virus, fowl mite and *Salmonella*), there was evidence of innate antibody binding to PAMPs. For *P. canariensis* fly extract, a faint band was visible around 15 kDa. Binding to fowl pox virus was detected with prominent bands at 15, 37 and 75 kDa. Less intense bands were visible around

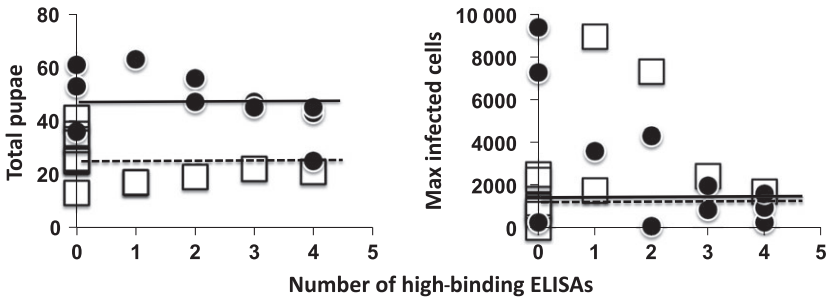

Figure 3 Antigen-binding diversity and parasite fitness. In each of 5 ELISAs used to measure antigen binding (see Figure 2), the samples that were in the upper quartile of optical density values were considered high binding. For each bird, the high-binding ELISAs were summed as a measure of antigen-binding diversity (i.e. more high-binding values = greater antigen-binding diversity). The counts of fly pupae (left panel) and malaria-infected cells (right panel) are shown relative to the number of high-binding ELISAs (*x*-axes). Samples are differentiated for birds that were bitted (closed circles) or not bitted (open squares) to control preening. On each panel, horizontal lines indicate median parasite counts for bitted (solid line) and nonbitted (dashed line) birds.

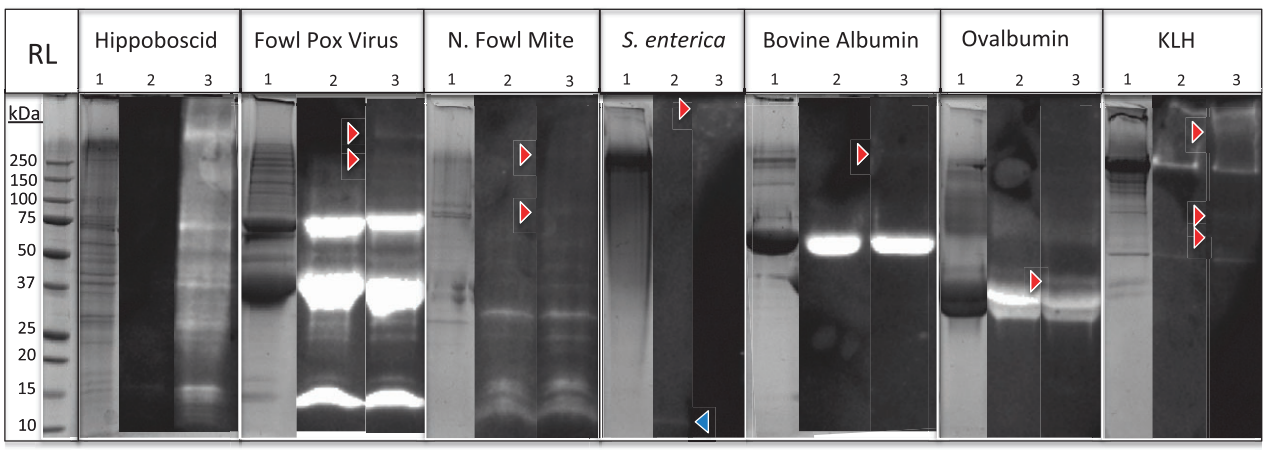

Figure 4 Antigen proteins and immunoblots. The assay proteins (column headings) were separated by electrophoresis on a 10–20% gradient polyacrylamide gel, stained with Coomassie, or transferred to PVDF membranes. Transferred proteins were incubated with pooled serum, probed with HRP-labelled anti-bird IgY antibodies. Binding was detected via chemiluminescence. The reference ladder (RL) shows relative sizes of proteins. For each extract, three images depict (1) molecule fractions in the extract vs. (2) antibody binding from pooled pre-infection serum and (3) pooled post-infection serum. Antibody binding to hippoboscid proteins increased dramatically after exposure to the flies (lane 3). Post-infection samples revealed new binding (red arrows) among antigens that were not involved with the parasite infections, suggesting that antigen binding diversified after host exposure to specific parasites. In one case (*S. enterica*), a detected band in pre-infection serum disappeared with post-infection serum (blue arrow). Matching bands in corresponding pre- vs. post-infection blots did not differ in intensity, indicating there was no change in the quantity of Ig binding to those bands.

25 and 50 kDa. These bands are similar to binding patterns observed with avian pox viruses from poultry and wild birds (23, 24), suggesting the antigens may be molecular patterns targeted by innate antibodies in pigeons. Similarly, bands were observed between 10 and 37 kDa with pre-infection serum and northern fowl mite extracts that correspond to bands observed with immunoblot analyses of chickens infested with that ectoparasite (25, 26). Only a single band was observed with pre-infection serum incubated against *S. enterica* extracts. The band, slightly larger than 10 kDa, corresponds to a band observed from chickens infected with *S. enterica* (27).

DISCUSSION

Antibodies are vital components of vertebrate immune defence against parasites, because they bind to molecular features of parasites and facilitate neutralization, or removal of the infection. Importantly, antibodies in a naive (uninfected) host must bind to antigens that have not been previously encountered to initiate antibody-mediated defences. This raises the question – what are the properties of pre-exposure antibodies that affect host responses to new parasite infections? One feature may be the ability of antibodies to bind molecular patterns

that are components of a parasite (PAMPs). Another feature may be the overall diversity of antigens that can be recognized by the repertoire of antibodies in circulation. These antibody traits are not mutually exclusive, but they do represent potentially different defensive effects. Binding to PAMPs is a focused defensive response to molecular targets that are conserved within a parasite taxon (e.g. lipopolysaccharide). Antibodies that bind diverse antigens (e.g. polyreactive Ig) may provide binding opportunities beyond PAMPs and facilitate recognition of rare parasites, or variable parasite antigens. Bindings to PAMPs and diverse antigens have both been associated with host–parasite interaction (14, 16). However, evidence for each has been derived from disparate studies that utilize different methodologies. As a result, it is unclear whether pre-exposure antibodies affect host–parasite interaction through specific PAMPs, or more broad antigen binding.

We tested the hypothesis that host antibody binding to antigens not previously experienced is predictive of subsequent host–parasite interactions. Using serum samples collected from host birds before infection with parasites, we measured total IgY and assayed for antigen binding by IgY. Importantly, the antigens were extracted from organisms never encountered by the host birds and the organisms represented parasitic and nonparasitic life histories. Thus, the assays included potential PAMPs and random molecular targets. We found that total IgY in serum before pathogen exposure was predictive of hippoboscid fly reproduction, but not *Haemoproteus* replication. Thus, total IgY was informative for one parasite, but not another. Similarly, IgY binding to extracts from multiple organisms did not uniformly predict parasite replication after infection. Individuals with higher levels of IgY binding to extracts from hippoboscids, *S. enterica* and adjuvant proteins had lower numbers of total fly pupae at the end of the experiment. Binding to extracts from northern fowl mites or fowl pox virus was uninformative. High binding to adjuvant proteins and extracts from hippoboscids was predictive of lower *Haemoproteus* replication, whereas binding to pox virus portrayed the opposite trend. Binding to extracts from mites and *S. enterica* was not predictive of *Haemoproteus* replication.

Among the assays, we observed evidence of Ig binding to PAMPs from ectoparasitic mites, avian pox virus and *Salmonella* bacteria. Immunoblots with chickens infected by these same parasites revealed antibody binding to antigens of similar molecular sizes to those we observed on immunoblots with pigeon serum (23–27). Importantly, the pigeons in this study were hatched and captive-reared with no exposure to any of these parasites. Thus, these antigens may represent conserved PAMPs that are innately recognized by pigeons. Unfortunately, there are no published immunoblot data for *H. columbae* flies, or closely related hippoboscids that would allow us to assess potential PAMPs of that group of ectoparasites. We also observed Ig binding to antigens unassociated with parasites. The immunoblots separately assayed binding to protein fractions of bovine serum albumin (cattle), ovalbumin (chicken) and haemocyanin (limpet). Earlier studies of antibody responses to these materials in poultry revealed similar binding patterns (28). These results support the idea that innate antibodies are polyreactive and not confined to PAMPs (21).

Effects of pre-exposure antibodies on host–parasite interaction have been described for a variety of parasites, including viruses, bacteria, protozoa, nematodes and arthropods (29–33) However, the evidence for these effects is split between two distinct types of study. In laboratory models using inbred mouse lines and *in vitro* cell cultures, there is evidence that mammals produce innate Ig that will bind to PAMPs. For example, McCoy *et al.* (32) demonstrated that naive mice possess IgG antibodies that bind nematode-associated proteins. Through transfer of serum between mouse lines, the authors showed that antibodies from naive mice conferred anti-nematode effects to knockout mice that were unable to produce their own antibodies. In field studies of wild species, researchers have characterized antibody-mediated immunity using *in vitro* HHL assays that evaluate Ig binding to multiple antigens from nonparasitic organisms. In contrast to laboratory model experiments, such field studies do not identify specific antigenic molecules or the isotypes of Ig involved with binding. Instead, those studies assume the assay antigens will be predominantly bound by innate, polyreactive immunoglobulins (16). Interestingly, there is evidence that HHL measures are related to parasitism in wild animals (16, 33–35). For example, recent experiments with wild boars showed that piglet antibody binding to chicken red blood cells was negatively correlated with infection by classical swine fever virus, implicating a protective effect of diverse antigen binding in viral infections of wild populations (33). Thus, laboratory and field studies implicate different properties of pre-exposure antibodies in host–parasite interaction. Laboratory experiments show innate Ig binding to PAMPs, and field data illustrate Ig binding to diverse antigens that are unassociated with parasites. The current study conclusively demonstrates that both PAMP and diverse, non-parasite antigen binding are predictive of host interactions with an ectoparasite, and a co-infecting protozoan parasite vectored by the ectoparasite.

We were not able to distinguish natural vs. adaptive antibodies in this study, because we did not isolate, nor track B-1 vs. B-2 lymphocytes. Given that the host birds were raised in captivity and that parasite exposure was strictly controlled, there is a high likelihood that pre-infection serum samples were dominated by innate antibodies. In contrast, the post-infection serum samples likely included adaptive antibodies produced in response to both the flies and the *Haemoproteus*. Regardless of these potential differences in antibody composition, we observed binding of antibodies to un-experienced antigens in both pre- and post-infection samples. Moreover, we observed higher levels of Ig binding to all extracts after parasite exposure. Immunoblots revealed new bands post-infection that were not present with blots by pre-infection serum. Those results suggest that the adaptive immune responses to flies and *Haemoproteus* diversified the antigen binding by circulating antibodies. Other studies have also shown that immune responses to pathogens affect the diversity of antigen binding (5, 14). For example, infection with human immunodeficiency virus stimulates the production of polyreactive antibodies that bind to host and bacterial antigens (36).

Parmentier *et al.* (37) observed that levels of innate Ig binding to *S. enterica* and *Staphylococcus aureus* PAMPs increased when hosts were challenged with antigens other than the PAMPs from those respective bacteria. One explanation for this phenomenon is that upregulation of humoral immunity during infection elevates production of antibodies unrelated to the infection. For example, in an experiment with transgenic mice, McCoy *et al.* (32) demonstrated that host infection with parasitic nematodes stimulated the production of antibodies specific to vesicular stomatitis virus, although the mice were unexposed to the virus. Another possible explanation is that adaptive immune responses to parasites include production of polyreactive antibodies. Our data support this possibility. With the exception of *S. enterica*, the bands detected with pre-infection serum were also detected with post-infection serum across all extracts. Those shared bands did not intensify with post-infection serum, as would happen if broad antibody production was elevated. Instead, new bands appeared that indicated novel antibodies were produced in response to parasitism and those antibodies were cross-reactive, or polyreactive.

Overall, our data indicate that the diversity of antigen binding is an important predictor of host–parasite interaction, whether the antigens are parasite-associated or not. Birds that had high levels of binding to multiple sources of antigens had lower parasite burdens (Figure 2). Although our results showed that higher and more diverse antigen binding predicted lower parasite fitness, it is critical to avoid the conclusion that antigen binding is always predictive of better defence. A higher immune response does not by default equate to better defence. For example, nonspecific human IgM has been shown to bind *Plasmodium falciparum* and subsequently protect the parasite from phagocytosis, potentially contributing to infection (38). Many pathological effects of parasites are attributed to damage caused by the host's immune response (immunopathology) (1, 39). Finally, given the ability of parasites to manipulate host immune function (see below) or avoid recognition (e.g. antigenic variation), it is also entirely possible for a given immune effector to have no impact on a parasite.

Our own data also suggest that antigen binding is not always predictive of host defence. For example, although we detected binding to antigens from northern fowl mites, the intensity of binding to that extract was not a significant predictor of fitness for either the fly or for *Haemoproteus*. Binding to pox virus antigens was unrelated to fly reproduction, and higher pox binding trended with higher *Haemoproteus* infections, suggesting a positive effect on protozoan fitness. Finally, in all the assays, we observed examples of low parasite fecundity on animals with low antigen binding (Figure 2). In other words, some parasites may benefit from high levels of particular antibodies or may reproduce poorly on animals irrespective of the hosts Ig properties.

Another caveat to consider is the potential for Ig profiles to change because of host–parasite interaction. We observed that pre-exposure Ig binding to fly proteins was negatively correlated with fly reproduction, but the post-exposure (adaptive) antibody binding to fly proteins was unrelated to fly fitness (20). This draws attention to the immunological processes that unfold during parasitism. For example, with blood-feeding arthropods, the host is exposed to antigens at the feeding site where mouthparts and saliva are introduced into host tissues. The saliva contains a multitude of effector molecules that can modulate host immune responses (40). These immunomodulators may negate antibody-mediated defences, regardless of antigen binding. For example, the blood-feeding fly *Stomoxys calcitrans* produces a salivary molecule that is strongly immunogenic and also binds the Fc region of host IgG (41). As a result, the salivary antigen stimulates and captures host IgG, which may protect the parasite from antibody-mediated defence.

Moreover, parasite antigens may be available (exposed) to the host immune response, but not engaged equally. Some parasite antigens may be immunodominant and stimulate strong immune responses while other antigens are not engaged (39). For example, the salivary glands and saliva of Tsetse flies (*Glossina* sp.) contain as many as 250

proteins potentially associated with blood feeding (42). In both laboratory animal models and samples from human populations, host antibodies bind to only a subset of molecules in the *Glossina* saliva (43). Some of those salivary antigens consistently stimulate strong antibody responses among all hosts, whereas others stimulate weak responses that vary among individuals (44). Molecules from a parasite may be effectively ignored by the adaptive antibody response, or highly targeted; the consequences of antibody binding to parasite antigens are not always clear. These examples illustrate that antigen binding is not inherently predictive of host defence, or effects on parasite fitness.

From an ecological perspective, the data we report create a mixed picture for future efforts to determine immunological interactions between wild hosts and parasites. Our results suggest that diverse antigen binding can be predictive of parasite infection, which validates innate antigen-binding assays (e.g. HHL) as tools for measuring immunological function and infection risk among individuals. However, the use of these assays is based on the expectation that innate antibodies are not affected by parasite exposure and are relatively independent of past infection (16). The results of our study are inconsistent with that assumption. Our data show that parasite exposure influences the diversity of antigen binding to both parasite-associated and unassociated molecules. Measures of innate antibodies from animals in the field should not be treated as independent of past exposure to antigens, or infection. In experiments where exposure history is uncontrolled, or unknown, relationships between innate Ig, host condition or host–parasite interaction could be confounded and should be treated with caution. Ideally, future work will characterize the Ig isotypes involved in the HHL assay and the blood cell antigens that stimulate binding. This would enable better alignment of field data with data from laboratory models. Researchers may also employ a panel of binding assays that encompass potential PAMPs and generic antigens as we have performed. This would fit with the multifaceted approach to immune function that has been proposed by several researchers (45–47).

In summary, our study confirms that an uninfected host (pre-exposure) produces antibodies that bind to parasite-associated antigens of multiple parasite taxa, as well as molecules derived from nonparasitic organisms. Binding to antigens that were not involved with an infection was predictive of parasite fitness after infection, regardless of the antigen's identity as a putative PAMP, or a generic foreign molecule. However, not all antigens were equally predictive of host–parasite interaction, and relationships between pre-exposure Ig and parasites differed among parasite taxa. Hosts that produced high levels of binding to diverse antigens consistently had fewer parasites, and the greatest parasite loads were observed on hosts with low levels of antigen binding. These patterns suggest that pre-exposure antigen binding is predictive of which hosts will produce many, parasites, vs. few parasites, following infection.

Finally, parasite infection appeared to increase the diversity of antigens bound by circulating antibodies, suggesting that the pre-exposure Ig repertoire is flexible and responsive to immunological challenges. These data raise intriguing questions at different levels of inquiry that may shape future research. At the molecular level, it will be important to determine what circulating antibodies are binding to parasite-associated antigens vs. generic foreign antigens. Are the antibodies that bind generic molecules also binding common PAMPs, or do they interact with other molecular targets that are uncommon, or less immunogenic? At the immunological level, it will be essential to determine why these antibodies are protective and what immune response(s) they facilitate. More broadly, how do these antibodies affect parasite resistance vs. parasite tolerance? At the physiological level, it will be important to learn whether these antibodies reflect the trade-offs between immune function and other physiological demands (e.g. growth), or responses to physiological stress. Finally, at the ecological level, it will be important to determine how past infection may alter immunological responses to new parasites and whether the Ig repertoire remains predictive of future host–parasite interactions. Answers to these questions should improve our understanding of Ig properties that impact host–parasite interaction and strengthen our ability to explore these phenomena among diverse wild species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Additional Methods.

