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Variation in the innate and acquired arms of the immune system among five shorebird species

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Summary

To contribute to an understanding of the evolutionary processes that shape variation in immune responses, we compared several components of the innate and acquired arms of the immune system in five related, but ecologically diverse, migratory shorebirds (ruff *Philomachus pugnax* L., ruddy turnstone *Arenaria interpres* L., bar-tailed godwit *Limosa lapponica* L., sanderling *Calidris alba* Pallas and red knot *C. canutus* L.). We used a hemolysis–hemagglutination assay in free-living shorebirds to assess two of the innate components (natural antibodies and complement-mediated lysis), and a modified quantitative enzyme–linked immunosorbent assay in birds held in captivity to assess the acquired component (humoral antibodies against tetanus and diphtheria toxoid) of immunity. Ruddy turnstones showed the highest levels of both innate and acquired immune responses. We suggest that turnstones could have evolved strong immune responses because they scavenge among rotting organic material on the seashore, where they might be exposed to a particularly broad range of pathogens. Although ruffs stand out among shorebirds in having a high prevalence of avian malaria, they do not exhibit higher immune response levels. Our results indicate that relationships between immune response and infection are not likely to follow a broad general pattern, but instead depend on type of parasite exposure, among other factors.

Key words: complement, habitat selection, humoral response, immunocompetence, immunoecology, natural antibodies, scavenging, shorebirds, wetlands, wildlife disease.

Introduction

The immune system is one of the most important defense mechanisms of vertebrates for protection against pathogens and parasites (e.g. see Zuk and Stoehr, 2002; Schmid-Hempel, 2003). Besides the obvious benefits, immune responses also convey costs, including greater risk of autoimmune disease (Råberg et al., 1998; Finch and Crimmins, 2004) and the depletion of energy that could otherwise be used in other activities (Nelson et al., 2002). Such costs, which potentially even reduce survival (Hanssen et al., 2004), will mould the evolution of the immune defence (e.g. Råberg et al., 2000). Therefore, maximising parasite resistance must be balanced by minimising damage to the host (Råberg et al., 1998; Segel and Bar-Or, 1999). This benefit/cost balance should depend on environmental conditions. For instance, relative benefits will increase with parasite density or parasite diversity (Råberg, 2002), while in habitats with high rates of infection, repeated activation of the immune response might select for strategies that minimise the risk of collateral damage and place a premium on optimising the immune responses (Segel and Bar-Or, 1999). The balance between benefit and cost is likely to lead to variation in immune response and indeed, within individuals of the same species, the immune function can vary with sex, age and season (Hasselquist et al., 1999; Duffy et al., 2000; Lorenzo and Lank, 2003; Nelson et al., 2002). In comparisons between species, immune response variation may also reflect the optimization of phenotype responses to the environment (Ricklefs and Wikelski, 2002); variation among species might thus represent phenotypic plasticity or genotype–environment interactions.

In vertebrates, the immune system consists of two arms, a non-specific, innate arm and a more specific, acquired arm (Male and Roitt, 2000; Doan et al., 2005). The innate immune system provides initial protection to a wide variety of foreign

organisms. The acquired immune system confers delayed, but more specific, protection against foreign antigens; in the blood stream it acts through specific antibodies that attach to its target pathogen. Higher levels of one component of the immune system need not imply greater overall resistance (Adamo, 2004); hence one should strive to assay the different parts of the immune system. In the present study, we collected several measurements of both the innate and the acquired (humoral) arm of the immune system.

Migratory shorebirds share many of the life-history traits that are thought to correlate with well-developed immune response, such as low reproductive rate and relatively long lifespan (Tella et al., 2002). However, this group of birds also varies with respect to migration strategy, habitat choice and foraging style (Piersma, 2003). While migration strategies might affect immune response through competition for limited energy resources (Piersma, 1997; Møller and Erritzøe, 1998), habitat choice also can create differences in disease risk (Moore, 2002; Mendes et al., 2005). In effect, while positive relationships between disease risk and immune response have been found in several studies (Lindström et al., 2004; Apanius et al., 2000), the relationship between migration and immunity may prove to be more difficult to uncover.

In this study, we use a combination of immunological assays that measure different branches of the immune system (innate as well as acquired) in a comparative and experimental study of five related Scolopacidae, including four Arctic-breeding and coastal wintering species: red knot *Calidris canutus* Linnaeus 1758, bar-tailed godwit *Limosa lapponica* Linnaeus 1758, sanderling *Calidris alba* Pallas 1764 and ruddy turnstone *Arenaria interpres* Linnaeus 1758, and the temperate-breeding ruff *Philomachus pugnax* Linnaeus 1758. Unlike the other species, the ruff is confined to freshwater wetlands year-round. Ruddy turnstones breed at more southerly latitudes than the other marine wintering species and they routinely scavenge among human and other refuse along seashores (Piersma et al., 1996). Among coastal shorebirds, ruddy turnstones seem to be particularly affected by wildlife diseases (Hansson, 2003), as are species using freshwater habitats in the tropics, such as ruff (Mendes et al., 2005).

Materials and methods

Wild shorebirds were caught along the East Atlantic flyway (Smit and Piersma, 1987; van de Kam et al., 2004). Coastal/marine shorebirds were caught at night with mistnets in the Parc National du Banc d'Arguin, northern Mauritania, ca. 20°N,16°W, during November–December 2002 and in the western Wadden Sea, The Netherlands, 53°,5°E, between 1999 and 2002 during northward and southward migration, and also during winter. In addition, we captured birds during the day using so-called 'wilsternets' (see Jukema et al., 2001) in the meadows of the Dutch province of Fryslân (ca. 53°N, 5°30′E) in April–May 2002. In total, we caught 54 red knots, 33 sanderlings, 15 ruddy turnstones, 8 bar-tailed godwits and 12 ruffs. Birds captured with wilsternets were bled within ca. 10 min after capture; those captured in mistnets within ca. 3 h.

Individuals of all five species to be held in captivity were caught in The Netherlands during the nonbreeding season. Three species were caught with mistnets at night during southward migration in the western Wadden Sea (53°16′N; 5°08′E): 10 red knots of the African wintering subspecies *C. c. canutus* and 11 sanderlings in July–August 2001, and two sets of ruddy turnstones, the first group with 24 individuals during August 2001 and the second with 11 individuals during November 2002, after post-breeding moult in the Wadden Sea (Meltofte et al., 1994). Fourteen bar-tailed godwits and ten ruffs were trapped with wilsternets in daytime during northward migration (Jukema et al., 2001). The bar-tailed godwits were caught in meadows on the island of Texel (53°05′N, 4°75′E) in May 2001, and the ruffs, in the province of Fryslân during April–May 2003. All birds were individually ringed, measured, weighed and aged as being in their first year of life or older on the basis of plumage characteristics (Prater et al., 1977).

Measuring immune responses

We chose assays to examine both the innate and the acquired arms of the immune system. Innate immunity was investigated in free-living individuals by measuring two of its most important components, i.e. natural antibodies and the complement cascade (Matson et al., 2005). Natural antibodies recognise and attach to invading organisms and are also responsible for initiating the complement cascade (Ochsenbein and Zinkernagel, 2000). The complement cascade recognises and kills extracellular foreign organisms (Wilson et al., 2002). To assess the acquired immune response, we challenged wild birds kept under identical aviary conditions with two antigens widely used in immunoecology studies, i.e. tetanus and diphtheria toxoid (inactivated toxin; e.g. Svensson et al., 1998; Råberg et al., 2003; Hanssen et al., 2004). In the present study, we considered antibody binding separately before vaccination and after primary and secondary immune responses, because these involve different mechanisms and molecules (Doan et al., 2005). In the humoral immune response, specific antibodies are responsible for neutralizing the intracellular pathogens by blocking cell binding/entry and preventing the spread of pathogenic organisms; they also neutralize toxins produced by bacteria such as diphtheria and tetanus (Roitt et al., 2000).

Hemolysis–hemagglutination assay in free-living shorebirds

A blood sample of ca. 160 μl was obtained by puncturing the brachial vein of wild shorebirds with a sterile 23-gauge needle; blood was collected in two 80 μl heparinized microhematocrit capillary tubes. Samples were stored on ice and were centrifuged for 10 min at 6900 ***g*** within 2 h. Plasma was stored at –20°C until analysis at the University of Missouri-St Louis.

To estimate the levels of circulating natural antibodies and complement we used the hemolysis–hemagglutination assay described in detail by Matson et al. (2005). The agglutination

reaction measures the interaction between natural antibodies and antigens, which results in blood clumping. The lytic reaction measures the amount of hemoglobulin released from the lysis of exogenous erythrocytes (e.g. rabbit), which is a function of the amount of lytic complement proteins present in the sampled blood. In both cases, quantification is achieved by serial dilution of plasma samples and assessment of the dilution step at which either the agglutination or lysis reaction stopped. For this assay, we placed 25 μl of plasma in six of the eight wells of the first row of a 96-well polysterene plate (Corning Costar #3795, Corning, NY, USA; 8 columns by 12 rows). The same amount of 0.01 mol l^{-1} sterile phosphate solution (PBS; Sigma #P3813, St Louis, MO, USA) was set in the first well to serve as the negative control; 25 μl of plasma of a well-known high responder (a chicken standard sample) was added to the last well as a positive control. Next, we used a multi-channel pipette to dilute with PBS all six plasma samples, the negative control and the positive standard sample up to 1:1024, through a set of ten 1:2 serial dilutions. After the addition of 25 μl of 1% of rabbit blood cell suspension to each well, each plate was sealed with a polystyrene plate lid. Plates were vortexed for 10 s at a low speed, and set to incubate at 37°C for 90 min. After incubation plates were tilted at a 45° angle along their long axis for 20 min at room temperature, then plates were scanned (Microtek Scanmaker 5900, Carson, CA, USA) using the positive transparency (top-lit) option and a full-size image (300 d.p.i.). We then quantified agglutination (which gives a measure of natural antibody levels) and complement-mediated lysis by assessing the dilution stage (on a scale from 1 to 12) at which these two reactions stopped (for further details, see Matson et al., 2005).

Humoral immune assays on wild birds held in captivity

With the exception of the 24 ruddy turnstones caught during August 2001 that were challenged with antigens 5 months after capture; all other birds were challenged within a month of capture.

To avoid the possibility of confounding effects of sex and age on the immune response, we attempted to restrict our experimental animals to adult females. Upon capture we selected bar-tailed godwits with the longest bills (Piersma and Jukema, 1990), red knots and sanderlings with long bills and the clearest brood patches (Nebel et al., 2000), and small-sized ruffs (van Rhijn, 1991). There are no external criteria for distinguishing female ruddy turnstones, and therefore we determined sex by a molecular PCR–DNA technique verified for red knots (Baker et al., 1999), and tested for sex and age differences in the group with enough individuals to compare between sexes or ages, the first group of ruddy turnstones (9 males and 15 females; 10 adults and 14 juveniles). We found no differences in diphtheria antibody levels between males and females or between first year and older birds (sex: repeated-measures ANOVA: $F_{1,20}$=0.13; P=0.73; age: $F_{1,20}$=0.29; P=0.60; sex×age: $F_{1,20}$=1.22; P=0.28) or in tetanus antibody levels (sex: repeated-measures ANOVA: $F_{1,20}$=0.11; P=0.75; age: $F_{1,20}$=0.63; P=0.44; sex×age: $F_{1,20}$=0.42; P=0.52). Therefore, in the context of interspecific comparisons, sex and age differences in antibody production are probably negligible.

Birds were kept in single-species flocks in large aviaries at the Royal Netherlands Institute for Sea Research (NIOZ) under the ambient natural light:dark cycle. The size of the aviaries, which had running freshwater and seawater, ranged from 1 m×3 m and 2.5 m high, to 7 m×7 m and 3.5 m high. Bar-tailed godwits, red knots, sanderlings and ruddy turnstones were fed trout pellets *ad libitum*, and ruffs also received mealworms *Tenebrio* sp. By 2 weeks after capture, body mass had stabilised and we presumed that birds had acclimated to captivity. At the time of testing, body masses as a percentage of the level at capture were 81±11% for bar-tailed godwits (mean capture mass=316 g, N=14), 87±11% for red knots (mean=137 g, N=10), 86±14% for sanderlings (mean=52 g, N=11), 90±15% for the first group of ruddy turnstones (mean=117 g, N=24), 98±14% for the second group of ruddy turnstones (mean=114 g, N=11), and 98±8% for the ruffs (mean =108 g, N=10).

Primary immune responses were elicited through vaccination with 120 μl of the combined tetanus and diphtheria toxoid in the pectoral muscle using a 0.5 ml sterile syringe (for further details of procedures, see Hasselquist et al., 2001). Secondary immune responses were elicited through a second vaccination with 100 μl of the same vaccine combination. Blood samples were taken prior to the first injection, and with the exception of the second group of ruddy turnstone, which were sampled 1 week later, at day 14 after the first injection and day 7 after the second injection, respectively (Feldman, 2000; Hasselquist et al., 1999, 2001; Owen-Ashley et al., 2004). Blood was centrifuged for 12 min at 6900 ***g*** and the plasma preserved at –30°C until analysis.

Antibody levels against tetanus and diphtheria toxoid were determined by using a modified quantitative enzyme–linked immunosorbent assay (ELISA; Hasselquist et al., 2001). Individual polysterene 96-well plates (Costar) were coated with either a diphtheria toxoid or with a tetanus toxoid {both diluted to 3 μg ml^{-1} with 0.06 mol l^{-1} of carbonate buffer [37 ml $NaHCO_3$ (1 mol l^{-1}) mixed with 13 ml Na_2CO_3 (1 mol l^{-1}) diluted in dH_2O to a total volume of 200 ml], at pH 9.6} and left to incubate overnight at 4°C. After washing three times with a buffer (0.01 mol l^{-1} PBS containing 0.05% Tween 20), all plates were blocked with 3% milk powder, diluted in the same buffer, for 2 h at room temperature. Plates were then washed twice and 100 μl of a 1:1600 diluted plasma sample was added (plasma was diluted in a 1:2 serial dilution with 1% milk powder mixed in PBS/Tween20) and left incubating overnight at 4°C. After three buffer washes, 100 μl of a 1:1000 diluted rabbit anti-passerine Ig antibody (produced against redwinged blackbird *Agelaius phoeniceus* antibodies; Hasselquist et al., 1999) was added to the wells and left to incubate for 1 h at 37°C. Plates were washed again two times and a diluted peroxidase-labelled goat anti-rabbit antibody (Catalogue no. A 6154, Sigma) was added and incubated for 30 min at 37°C. Plates were washed twice and thereafter the

substrate solution [200 μl of 0.2 mmol l^{-1} ABTS (Catalogue no. A 1888, Sigma) and 80 μl of 30% H_2O_2 (diluted 1:40 in distilled H_2O) mixed in 20 ml of citrate buffer, pH 4.0] was added to achieve colour reaction. We used a Vmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) to read the kinetics of colour reactions at 405 nm every 30 s for 14 min. Calculation of antibody titers was based on the slope of the substrate conversion, in millioptical density units min^{-1} (mOD min^{-1}).

Statistical analysis

All samples from the specific antibody measurements were run in duplicate. Repeatability (intersample variability) was estimated as a percentage of the total variability; interplate variability was based on the series of diluted reference samples (1:600 to 1:76800) run on each plate. Intersample variability was 2% and interplate variability was 16%. We used the average values of the duplicate samples in all analyses. To account for interplate variation we adjusted all values to be comparable with a reference plate, using plasma from one red knot (known to be a high responder) as reference sample on all plates.

Natural antibody data were $\log_2$-transformed, to achieve normality (samples were 1:2 serial diluted). We tested for interspecific differences in natural antibody levels with analysis of covariance (ANCOVA), with body mass entered as a covariate. Complement activity data was not normally distributed, and therefore we used Kruskal–Wallis (multiple species) and Kolmogorov–Smirnov tests (two species), to test for interspecific differences (Sokal and Rohlf, 1995).

Humoral antibody titers were $\log_{10}$-transformed to normalize the residuals (Sokal and Rohlf, 1995). We accounted for the unwanted variability caused by interspecific differences in body mass by using an ANCOVA, with body mass entered as a covariate. Furthermore, to identify which species exhibited the highest antibody response, we performed a *post hoc* Tukey test.

To investigate whether immune responses exhibit a general pattern, we correlated the different immune measurements at the individual and the species levels. We used the parametric Pearson correlation coefficient to determine the relationships between complement activity and natural antibody levels (innate components) and between tetanus and diphtheria humoral response (acquired components). Because the innate and acquired measurements were taken in different individuals, we used Spearman rank correlations to see whether species average response values correlated among and between the two arms of the immune system. All tests were performed in SYSTAT 9 for Windows.

Results

Natural antibodies and complement activity of wild birds

Natural antibodies levels only differed among species when we corrected for body mass (ANCOVA: species $F_{4,121}$=1.41; P=0.23, body mass $F_{1,121}$=1.24; P=0.27; species×body mass $F_{4,121}$=2.63; P=0.04; Fig. 1). The level of complement activity varied significantly among species (Kruskal–Wallis U=43.36, d.f.=4; P=0; Fig. 1). The non-parametric Kolmogorov–Smirnov test revealed that ruddy turnstones had the highest level of complement-mediated lysis (all species: P<0.05; see also Fig. 1).

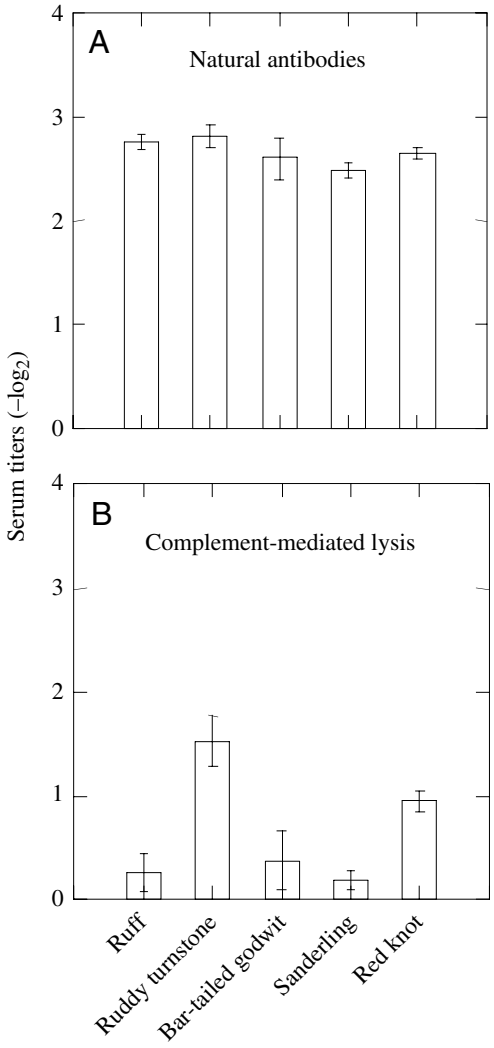

Fig. 1. (A) Natural antibody levels and (B) complement-mediated lysis in five species of shorebirds, estimated from the $\log_2$-transformation of the score of the 1:2 serial dilution of the shorebirds' sera. Natural antibody levels were calculated at the step where agglutination stops and complement at the step at which lysis stops (see text for details). Values are means ± s.e.m.

Humoral immune assays on wild birds held in captivity

The two groups of ruddy turnstones differed with respect to diphtheria pre-vaccination antibody levels (ANCOVA: trial $F_{1,31}$=6.40, P=0.02; body mass $F_{1,31}$=2.06, P=0.16) and tetanus primary immune response (ANCOVA: trial $F_{1,31}$=4.92, P=0.03; body mass $F_{1,31}$=0.15, P=0.70), but not with respect to the primary immune response against the diphtheria toxoid (ANCOVA: trial $F_{1,31}$=0.92, P=0.35; body mass $F_{1,31}$=0.41, P=0.53), or the secondary immune response (ANCOVA: trial

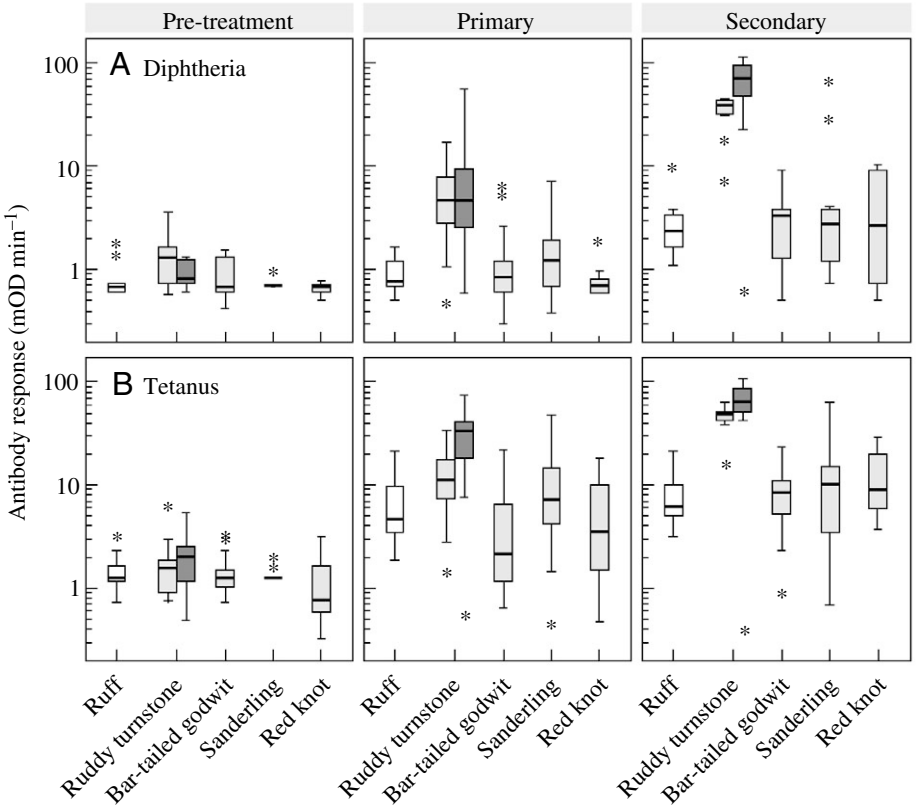

Fig. 2. Antibody titers before and after (repeated) vaccination with (A) diphtheria and (B) tetanus toxoids in five shorebird species tested within a month of capture. For the ruddy turnstone, the darker box represents the additional experiment where birds were tested 5 months after capture. The ruff, the only freshwater specialist, is indicated by a white box. The boxes enclose the 25% quartiles around the medium (horizontal line); the whiskers indicate the range of observed values that fall within a 1.5 spread of the interquartile range; and the asterisks represent observed values that fall outside that spread.

$F_{1,31}$=0.84, P=0.37; body mass $F_{1,31}$=0.13, P=0.73) (Fig. 2). The same was true for the pre-vaccination (ANCOVA: trial $F_{1,31}$=0.29, P=0.60; body mass $F_{1,31}$=0.27, P=0.61) and secondary antibody titers against the tetanus antigen (ANCOVA: trial $F_{1,31}$=0.05, P=0.82; body mass $F_{1,31}$=0.22, P=0.65). Although the absolute magnitudes of these differences were small compared to the differences between the shorebird species (Fig. 2), we nonetheless included only the group of ruddy turnstones that were challenged within a month of capture in the interspecific analysis.

All species responded positively to vaccination by producing antibodies against the diphtheria toxoid (repeated-measures ANOVA: ruff $F_{2,18}$=17.96, P=0; ruddy turnstone $F_{2,16}$=111.39, P=0; bar-tailed godwit $F_{2,26}$=12.39, P=0; sanderling $F_{2,20}$=8.93, P=0; red knot $F_{2,18}$=10.11, P=0) and the tetanus toxoid (repeated-measures ANOVA: ruff $F_{2,18}$=26.37, P=0; ruddy turnstone $F_{2,16}$=81.26, P=0; bar-tailed godwit $F_{2,26}$=18.26, P=0; sanderling $F_{2,20}$=14.44, P=0; red knot $F_{2,18}$=23.92, P=0; see also Fig. 2).

Diphtheria antibody levels differed between species, even before vaccination (ANCOVA: species $F_{4,9}$=4.54, P=0; body mass $F_{1,49}$=0.07, P=0.79). The interspecific differences in diphtheria antibody levels widened during the primary (ANCOVA: species $F_{4,47}$=6.23, P=0; body mass $F_{1,47}$=0.09, P=0.77) and the secondary immune responses (ANCOVA: species $F_{4,47}$=16.92, P=0; body mass $F_{1,47}$=2.95, P=0.09). In contrast, tetanus antibody levels did not differ between species, either before vaccination (ANCOVA: species $F_{4,49}$=1.06, P=0.39; body mass $F_{1,49}$=0.29, P=0.59), or during the primary immune response (ANCOVA: species $F_{4,47}$=0.90, P=0.47; body mass $F_{1,47}$=0.06 P=0.82), but they did differ during the secondary immune response (ANCOVA: species $F_{4,47}$=9.94, P=0; body mass $F_{1,47}$=2.68, P=0.11). *Post hoc* Tukey tests revealed that the ruddy turnstone had (in the case of diphtheria), or developed (in the case of tetanus), higher antibody levels to the same amount of vaccine than the other species. Pre-vaccination, primary and secondary antibody levels against diphtheria and secondary antibody levels against tetanus did not differ among the other species (see also Fig. 2).

Relation between the different immune measurements

The two innate components measured in this study, i.e. natural antibody level and complement-mediated lysis, were not correlated (r=0.09, N=127, P=0.17), but the two measurements of the acquired arm of the immune system (antibody titers against diphtheria and tetanus) were positively correlated during pre-injection (r=0.66, N=44, P=0), primary response (r=0.63, N=54, P=0) and especially secondary immune response (r=0.82, N=55, P=0).

Even though the correlations between innate and acquired immune components were based on the data points for the five species and were never significant at the 5% level, there was a tendency for a positive correlation between natural and background antibodies against diphtheria and between complement activity and secondary tetanus antibody titers (Table 1).

Table 1. *Spearman rank correlation coefficients (r_S) based on humoral immune response values, calculated from the species averages*

	Diphtheria			Tetanus		
	Background	Primary	Secondary	Background	Primary	Secondary
Natural antibodies	0.7	0.1	0.1	0.5	0.4	0.3
Complement-mediated lysis	0.3	0.1	0.4	0.4	0.1	0.7

All correlations were positive, but none were significant at the 5% confidence level; when $r_S>0.7$, then $0.05<P<0.1$ ($N=5$).

Discussion

Although we found considerable interspecific variation in both innate and humoral immune components, differences were most pronounced for complement-mediated lysis and primary and secondary humoral immune responses. This result suggests that not all immune components are under the same pressure to be internally regulated. Indeed, the levels of natural antibodies varied little, even among species with such different body masses as the sanderling and the bar-tailed godwit. This is consistent with the idea that natural antibody production is largely independent of internal and external stimuli (Ochsenbein and Zinkernagel, 2000). However, although natural antibodies are present in relatively low densities, they play an important role in the initial recognition of foreign particles and they support subsequent defense by the complement cascade and the acquired humoral response (Ochsenbein and Zinkernagel, 2000; Turner, 2000). Therefore, organisms may benefit by maintaining a minimum level of immunoglobulins, as these molecules likely convey benefits in terms of earlier detection of parasites. With respect to the innate immune system, we found no difference between the five shorebird species in natural antibody levels, whereas ruddy turnstones showed a higher complement system activity than the four other species. For the humoral responses of the acquired immune system, pre-injection, primary and secondary antibody titers against diphtheria toxoid and secondary antibody titers against tetanus were higher in ruddy turnstones, whereas there were no differences in antibody responses between any of the other shorebird species.

The hemolysis–hemagglutination assay measurements of natural antibodies and complement activity were well within the range of values found for other bird groups (Matson et al., 2005). With respect to the ELISA assay of antibody levels against tetanus and diphtheria, we found that the primary and secondary antibody titers in all five shorebird species were significantly higher than pre-injection values. Hence, despite the ELISA being designed for passerine birds, it apparently also works well in shorebirds. Among all five shorebird species, antibody responses against diphtheria were lower than those against tetanus, which is in accordance with other studies on wild birds (e.g. Westneat et al., 2003; Owen-Ashley et al., 2004).

We did not find any correlation between the two innate components (natural antibody level and complement-mediated lysis), nor between innate and acquired components. This result underlines the problem of obtaining a ‘general’ measure of immunocompetence and emphasizes the importance of measuring different aspects of the immune system (Adamo, 2004; Matson et al., 2005). There was a tendency for a relationship between natural antibodies and background antibody titers, which suggests that they both might reflect the basic level of (polyclonal) natural antibodies in the circulation.

Ruddy turnstones stand out as high responders in three of the four immune measurements taken (complement-mediated lysis, humoral responses to tetanus and diphtheria toxoid). This difference is not likely to be explained by phylogeny because turnstone’s closest relatives (sanderling, red knot and ruff) were as low responders as the more distantly related bar-tailed godwit (see Piersma et al., 1996). Thus, the high responder is embedded in a clade of low responders in our study, and presumably evolved from a low-response state. Furthermore, neither habitat choice *per se* nor migration strategy can explain the exceptionally strong immune responses observed in the ruddy turnstone, since this species shares coastal wetlands and long-distance migration with other low responders, such as the bar-tailed godwit, the sanderling and the red knot. Ruddy turnstones do stand out, however, by their scavenging habits. They often feed on decomposing food remains, including dead fish and mammals (Piersma et al., 1996), and as a consequence they are often found close to human settlements, e.g. in harbours, where they are likely to benefit from an abundance of such food items. This opportunistic feeding style might expose them to infections, particularly diseases that are transmitted by contaminated dead animals, e.g. Avian Cholera or Herpes virus (Friend and Franson, 2001). Indeed, in the eastern USA, ruddy turnstones carried 67.5% of Avian Influenza Virus (AIV) infections, even though they accounted only for 12.4% of 2162 individuals from 15 different shorebird species in a study by Hansson (2003).

We suggest that in the nonbreeding season ruddy turnstones might be exposed to a particularly broad range of disease organisms, and that they therefore require high responsiveness in several parts of the immune system. A similar conjecture was made for populations of the Darwin’s finch *Geospiza fuliginosa*, in which islands with the highest prevalence of avian pox and feather mites supported host populations with the highest natural and humoral immune responses (antibody levels; Lindström et al., 2004).

It is perhaps surprising that ruffs exhibited low levels of immune response, as they occur in inland freshwater habitats

where the likelihood of avian malaria infection is high (Mendes et al., 2005). This environment presumably would select ruffs to invest strongly in their immune systems (Piersma, 1997), but this hypothesis was not supported here. Note, however, that we did not measure cell-mediated immunity, a type of response known to be involved in the control of malaria parasites (Wakelin, 1998; Doan et al., 2005).

To the best of our knowledge, this is the first time that a suite of immune system measures has been applied to shorebirds in a comparative study of immunocompetence between species. In brief, our findings emphasize the need to study several immune components, preferably from different arms of the immune system, when assessing 'general immunocompetence'. Furthermore, we suggest that the relationships between immune response and infection patterns are particular, rather than general, and depend strongly on the range and strength of exposures and the precise variety of parasite types.

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