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RESEARCH ARTICLE

Flight performance of western sandpipers, *Calidris mauri*, remains uncompromised when mounting an acute phase immune response

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SUMMARY

Migratory birds have been implicated in the spread of some zoonotic diseases, but how well infected individuals can fly remains poorly understood. We used western sandpipers, *Calidris mauri*, to experimentally test whether flight is affected when long-distance migrants are mounting an immune response and whether migrants maintain immune defences during a flight in a wind tunnel. We measured five indicators of innate immunity in 'flown-healthy' birds (flying in a wind tunnel without mounting an immune response), 'flown-sick' birds (flying while mounting an acute phase response, which is part of induced innate immunity), and a non-flying control group ('not-flown'). Voluntary flight duration did not differ between flown-healthy and flown-sick birds, indicating that mounting an acute phase response to simulated infection did not hamper an individual's ability to fly for up to 3 h. However, in comparison to not-flown birds, bacterial killing ability of plasma was significantly reduced after flight in flown-sick birds. In flown-healthy birds, voluntary flight duration was positively correlated with bacterial killing ability and baseline haptoglobin concentration of the blood plasma measured 1–3 weeks before experimental flights, suggesting that high quality birds had strong immune systems and greater flight capacity. Our findings indicate that flight performance is not diminished by prior immune challenge, but that flight while mounting an acute phase response negatively affects other aspects of immune function. These findings have important implications for our understanding of the transmission of avian diseases, as they suggest that birds can still migrate while fighting an infection.

Key words: wind tunnel, innate immune function, trade-off, western sandpiper, *Calidris mauri*, zoonotic diseases.

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INTRODUCTION

Emerging infectious diseases in humans are dominated by zoonoses, diseases that are transmitted from animals to humans (Jones et al., 2008). Several zoonoses have migratory animals as their natural host, and it is often assumed that long-distance migrants can increase the spread of zoonotic pathogens (Reed et al., 2003; Leroy et al., 2009). However, as Ricklefs et al. (Ricklefs et al., 2005) succinctly pointed out, 'if migrants are such highly mobile sources of potential infection, why is it that they do not cause more disease epidemics than they appear to?' A possible explanation is that individuals infected with pathogens may not be capable of flying long distances (Hoye, 2011) because pathogens consume host resources (Price, 1980), damage host tissues (Read et al., 2008) or prompt the host to mount immune responses (Lochmiller and Deerenberg, 2000). Maintaining immune function and launching an immune response can be costly in terms of energy, nutrients, autoimmunity and oxidative stress (Klasing, 2004; Hasselquist and Nilsson, 2012), and as a result immune function may be subject to trade-offs with other energetically expensive activities (Ardia and Schat, 2008; Buehler et al., 2008; Owen and Moore, 2008).

A relationship between infection and strenuous exercise in migratory birds and other organisms has been suggested previously. For example, wild Bewick's swans, *Cygnus columbianus*, infected with low-pathogenic avian influenza showed delayed migration in comparison to uninfected birds (van Gils et al., 2007). In addition, performing strenuous exercise may in turn affect immune defences, potentially making organisms more susceptible to infection. This is particularly well documented in humans through the biomedical discipline of exercise immunology (Shephard, 2010): human athletes often experience reduced immune function during strenuous exercise (Harper Smith et al., 2011; Walsh et al., 2011). Likewise, a recent study on European starlings, *Sturnus vulgaris*, showed a negative relationship between strenuous exercise and immune function. Non-induced aspects of immune function (plasma levels of haptoglobin, agglutination and lysis) decreased significantly after flight in a wind tunnel (Nebel et al., 2012). The effects of strenuous exercise on immune function likely vary among different aspects of the immune system. For example, a study on red knots, *Calidris canutus*, found that induced humoral and cell-mediated responses were not affected by flight in a wind tunnel (Hasselquist et al., 2007). This highlights that the effects of flight on immune function – and *vice versa* – remain poorly understood (Matson et al., 2012).

We experimentally tested a predicted trade-off between immune function and flight in a wind tunnel by assessing whether flight performance is diminished when long-distance migrants are mounting an immune response and whether migrants can maintain immune defences during flight. We focused on innate immune function, the part of the vertebrate immune system that provides immediate and non-specific defence against a range of pathogens. Some aspects of innate immune function, such as the ability of

blood components to kill bacteria, are constantly maintained (though their levels may fluctuate depending on environmental circumstances). Others, such as the acute phase response, are present only when induced by a challenge. We measured levels of constantly maintained aspects of innate immune function and experimentally induced an acute phase response in western sandpipers, *Calidris mauri* Cabanis 1857, before and after flying in a wind tunnel. We induced an acute phase response by mimicking bacterial infection with an injection of lipopolysaccharide (LPS) (Bonneaud et al., 2003). This challenge is commonly used to elicit sickness behaviour and fever without causing actual disease, as no live bacteria are present. We addressed four questions: (1) does mounting an acute phase response diminish flight performance; (2) does flight diminish levels of constantly maintained aspects of innate immune function; (3) does flight diminish the acute phase response; and (4) is there a relationship between immune function and flight duration?

MATERIALS AND METHODS

Overview of experimental design and groups

We performed a series of tests to address the four questions outlined above. The first test was designed to determine the effect of an immune challenge on flight performance. To do so, we compared flight duration and speed in western sandpipers that received an LPS injection 1 day prior to flight ('flown-sick') with birds that were handled in the same way but did not receive an injection before flight ('flown-healthy'). We then wanted to determine the effect of flight on immune function, and compared flown-healthy and flown-sick birds with birds that were held in a cloth-covered carrier cage without food or water for the duration of the flight ('not-flown'). Finally, we injected both flown-healthy and not-flown birds with LPS within 7 min of their treatment (flight or rest), and compared the acute phase response between the two groups the following day. Note that the flown-sick birds were blood sampled only twice (baseline and after flight), whereas flown-healthy and not-flown birds were sampled three times (baseline, after flight or rest, and 1 day after flight or rest).

Bird capture and husbandry

All western sandpipers used in this experiment were captured on 29–30 April 2011 at Boundary Bay, British Columbia, Canada, and housed temporarily at aviaries at Simon Fraser University. Within a few days of capture, they were transported by air and vehicle to indoor shorebird aviaries at the Advanced Facility of Avian Research (AFAR) at Western University, London, Ontario, Canada. The aviaries have special soft floors to protect feet from calluses, and each room has a pool with continuously running freshwater. Experiments were conducted in August when sandpipers normally migrate to the wintering areas. Birds were on a photoperiod of 20 h:4 h light:dark, imitating the light conditions on the breeding grounds in Nome, Alaska. All birds were captured under Canadian Wildlife Service scientific collection permit CA-0256 and all animal procedures were approved by the Western University Animal Use Subcommittee (protocol 2010-216).

Diet and treatment against infection

Birds were offered a 50:50 diet of turkey chick starter/grower complete (Purina, Agribrands Purina Canada, Woodstock, ON, Canada) and fish pellets (Aqua Max, Fingerling Starter 300, pellet size: 1/16 inch; Gray Summit, MO, USA), but showed a strong preference for the latter. The diet was periodically supplemented with mealworms *Tenebrio molitor* and freeze-dried *Gammarus* shrimp (Turtle Snack, Nutrafin basix, Rolf C. Hagen, Baie d'Urfé, QC, Canada).

All experimental birds were treated on 21 May 2011 against infections with a single dosage (2 µl g^{-1} body mass) of Ivermectin (concentration: 10 mg ml^{-1}; Merial Canada, Baie d'Urfé, QC, Canada), a broad-spectrum antiparasitic. For 2 weeks, starting 20 May, they were given drinking water containing 0.024% amprol (concentration: 9.6%; Huvepharma, Mitchel, ON, Canada), a treatment for caecal coccidiosi, and a suspension of 500 mg l^{-1} metronidazole (Pfizer, Wellesley, ON, Canada), which protects against bacteria and protozoans. They were also injected subcutaneously with 60 µl droncit (concentration: 5.68 mg ml^{-1}; Bayer, Toronto, ON, Canada) against tapeworms on 23 June. Birds were treated topically with pyrethrin (Bird Bath, Living World, Rolf C. Hagen) against lice and feather mites on 2 August. All treatments were completed prior to the start of the experiment on 9 August. We cannot exclude the possibility that birds treated against infection respond differently to LPS because of altered host–parasite relationships, but as all birds in this study were treated in the same way, we are confident that any effect on flight performance or immune function we observe is due to the experimental treatment as opposed to a pre-existing infection.

Wind tunnel flight training

Birds were flown in a recirculating wind tunnel at AFAR (Gerson and Guglielmo, 2011). A fine mesh net was stretched tight across the front of the test section. Birds were flown at 15°C and 70% relative humidity at the maximum air speed (up to a maximum of 11.0 m s^{-1}) they appeared comfortable with (i.e. without abrupt changes in flight behaviour). Six weeks before the experimental flights all birds were screened for flight ability in the wind tunnel by S.N. and A.M. Each bird was given repeated trainings in the tunnel, where it was flown for up to 15 min in wind speeds between 5 and 11 m s^{-1}. Birds with poor flight performance during the training sessions were assigned to the control group (N=9; three females, six males), the others were assigned to the flight group. Individuals were randomly split between the flown-sick (N=9; three females, six males) and flown-healthy (N=9; one female, eight males) treatments. Thus, we only compared flight performance among birds that were willing to fly in the tunnel. While it is not impossible that birds that were assigned to the non-flying control group have poorer immune function than the better fliers, we are not aware of any study that has suggested such a relationship.

Detailed protocol and sampling regime

Approximately 15 days (range: 7–23 days) prior to the experimental flight, we took a blood sample to test for pre-existing differences in immune function among birds (Fig. 1). The day before their experimental flight, flown-sick birds were given an intraperitoneal

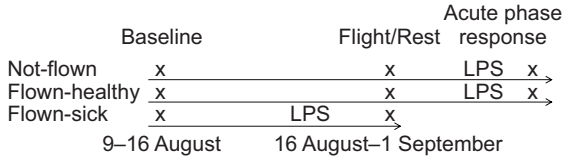

Fig. 1. Experimental setup. 'Flown-sick' western sandpipers (mounting an acute phase response during flight) were injected 23 h prior to flight, and 'not-flown' (no acute phase response, no flight) and 'flown-healthy' (not mounting an acute phase response during flight) birds were injected with lipopolysaccharide (LPS) immediately after flight. The final blood sample was taken 23 h afterwards.

injection of LPS (see below). Flown-healthy and not-flown birds were handled in the same way but did not receive an injection (Koutsos and Klasing, 2001). To ensure a post-absorptive state, all birds were fasted 2 h prior to the beginning of the flight/rest treatment. Both flown-sick and flown-healthy birds were introduced into the test section of the wind tunnel with an air speed of 5 m s^{-1}, at which point the time was recorded. Wind speed was increased until a wind speed was reached at which the bird was comfortable flying. Wind speed was decreased once a bird was blown backwards. Flight speed was recorded in 1 s intervals. During the first 10 min of the flight birds could land repeatedly. After these first 10 min, which counted towards total flight time, a flight was considered terminated once a bird landed, touched a catch net at the rear of the test section three times or refused to resume flight. Experimental treatment was unknown to the person conducting the flights. Three birds could be tested each day (one from each treatment), and the entire experiment lasted for 3 weeks (15 August to 2 September 2011).

Birds in the control treatment (not-flown) were held in a cloth-covered box for the duration of the flown birds' (healthy or sick) flight. This means that flown-healthy, flown-sick and not-flown groups may have experienced different levels of stress in the sense that flying in a wind tunnel may elicit more (or less) stress than sitting in a dark room. At the end of the experimental flight/rest each bird was blood sampled, and body mass and cloacal temperature were measured. We measured body mass using an electronic balance to the nearest 0.1 g and body temperature to the nearest 0.1°C using a fast-reading, high-accuracy thermocouple (VWR Scientific 23609-232, Radnor, PA, USA). To sample blood, we sterilized the area around the brachial vein with 70% ethanol and pricked the vein with a sterile 26 gauge needle. We collected ca. 150–200 µl blood into heparinized capillary tubes and then transferred the blood to 1.5 ml microcentrifuge tubes. Plasma was obtained by centrifuging blood samples for 10 min at 2000 ***g*** and was then stored for ~4 months at −80°C until processing.

Flown-healthy and not-flown birds were then injected with LPS and returned to their aviary. Twenty-three hours later, flown-healthy and not-flown birds were bled and weighed again, and cloacal temperature was measured. The timing of this measurement, at 23 h after LPS injection, was based on a pre-trial pilot experiment, where we assessed the effect of the LPS injection on the temperature response in hourly intervals (16–24 h). Birds that received an LPS injection had significantly higher body temperature 23 h post-injection than those that did not ($F_{1,6}$=7.45, P=0.034). A change in body temperature is indicative of the acute phase response, which is part of the induced innate immunity.

Immune assays

LPS injection

Birds received an intraperitoneal injection of LPS using a 23 gauge needle and a 1 ml syringe. We used a solution of 0.25 mg ml^{-1} LPS (Sigma L 7261, source strain *Salmonella typhimurium* ATCC 7823; Sigma-Aldrich, St Louis, MO, USA) in phosphate buffered saline (PBS) (Sigma-Aldrich) at a dosage of 1 mg kg^{-1}. This dosage was determined after a pre-trial of several lower dosages (0, 0.25, 0.50 and 0.75 mg ml^{-1} LPS) and has been used previously in species of similar size (Koutsos and Klasing, 2001; Buehler et al., 2009).

Bacterial killing ability

We assessed the antimicrobial capacity of plasma using spectrophotometry (Liebl and Martin, 2009). We used gram-negative bacteria *Escherichia coli* (ATCC 8739), which were supplied lyophilized as 10^7 or 10^8 organisms per pellet (E^{power} Assayed Microorganism Preparation, Microbiologics, Saint Cloud, MN, USA). They were reconstituted according to the manufacturer's instructions in 10 ml of tissue-culture-grade PBS. Cultures were diluted to 1×10^5 organisms ml^{-1}, and plasma was diluted 1:23 (1.5 µl plasma in 34.5 µl PBS). One-third of the sample volume of the solution (12.5 µl) containing the *E. coli* was added to diluted plasma, vortexed and incubated at 37°C for 30 min. After primary incubation, all of the samples were removed, vortexed and 250 µl of sterile tryptic soy broth (TSB) was added to each sample. Then, samples were again vortexed and subsequently incubated for 12 h at 37°C. We then added 12.5 µl of the inoculated working solution to 36 µl of sterile PBS and 250 µl of TSB; these served as positive controls.

Upon completion of the secondary incubation, the microbial concentrations were determined by measuring the absorbance of each sample at 300 nm using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). Sterile blanks were used as previously described, and the microbicidal activity of plasma was calculated as the proportion of microbes killed in samples relative to positive controls [1–(absorbance of sample/absorbance of control)] (Liebl and Martin, 2009).

Blood plasma proteins

We used the hemolysis-hemagglutination assay to measure complement-like and natural antibody activity, which provides early protection against infections *via* cell lysis (Ochsenbein and Zinkernagel, 2000). For this assay, bird plasma is mixed with rabbit red blood cells. The agglutination reaction measures the interaction between natural antibodies and antigens, which results in clumping of the rabbit red blood cells. The lytic reaction measures the lysis (disintegration) of rabbit red blood cells, 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 (Matson et al., 2005). Specifically, we placed 25 µl of plasma in the first two rows of a 96-well round-bottom plate. From row 2 to row 11, we performed ten 1:2 dilutions using Dulbecco's PBS. We then added 25 µl of 1% of rabbit red blood cell suspension to each well and placed the plates in a water bath at 37°C for 90 min. After incubation, we tilted the plates 45 deg and then scanned them (HP Scanjet G3110, Mississauga, ON, Canada) after 20 min for agglutination and after 90 min for lysis. The scans were scored blindly by a single observer (S.N.) using the criteria outlined in Matson et al. (Matson et al., 2005).

Haptoglobin is an acute phase protein that protects against harmful end-products of the immune response (Delers et al., 1988). Acute phase proteins limit injury and contribute to healing (Pickup, 2004). We quantified plasma haptoglobin levels following the 'manual method' in a commercial kit (no. TP801, Tri-Delta Diagnostics, Morris Plains, NJ, USA), which exploits the peroxidase activity of haptoglobin bound to haemoglobin.

Statistical analyses

We used a generalized linear model (GLM) with Type III sum of squares to assess whether flight duration (min) and average flight speed (m s^{-1}) differed between flown-healthy and flown-sick birds and whether levels of the immune indicators (bacterial killing, haptoglobin, lysis or agglutination) differed among the three experimental groups. We used Tukey's *post hoc* test to assess differences among groups. Sexes were pooled for analyses. A GLM was used to test whether the change in temperature and body mass (post-LPS values minus pre-LPS values) differed between flown-

healthy and not-flown birds. A GLM was also performed to test for correlation between flight duration and immune function in flown-healthy and flown-sick birds. We tested for such a correlation with baseline and post-flight values within both groups, and also in acute phase response values in flown-healthy birds. We used paired *t*-tests to test for the effect of mounting an acute phase response on all immune indicators in flown-healthy and not-flown birds. Values for lysis did not conform to normality, thus we used the Kruskal–Wallis test to detect differences in lysis levels among groups. We calculated the non-parametric rho, the Spearman rank-order correlation coefficient, and the corresponding two-tailed *P*-value to test for correlations between lysis and flight duration.

We acknowledge that measuring multiple aspects of immune function across different experimental groups to answer different questions entails multiple test of significance and the ensuing risk of erroneously interpreting some relationships as statistically significant (Type I error). For this reason we report exact *P*-values allowing readers to combine information presented in the *P*-values, means and graphs to judge the biological significance of our results. For simplicity, we interpret *P*-values less than 0.05 as statistically significant in the Results and Discussion. We have opted against using a Bonferroni-adjustment to control for multiple comparisons. This adjustment is now often viewed as overly conservative due to the drastic reduction in statistical power that it entails, and the difficulties in determining the number of comparisons for correction in complex research designs (Gotelli and Ellison, 2004; Nakagawa, 2004). Data are presented as means ± s.d.

RESULTS

Question 1: effect of an immune challenge on flight performance

There was no effect of mounting an acute phase response on flight duration or speed. The mean duration of experimental flights in flown-healthy birds was 55.1±54 min (range: 10–148 min, N=9), and the mean flight speed was 8.3±1.6 m s^{-1} (range: 5.6–10.5 m s^{-1}, N=8). In flown-sick birds, flight duration was slightly shorter (mean: 46.6±52.8 min, range: 0–180 min, N=9) but flight speed was the same as in flown-healthy birds (mean: 8.3±1.6 m s^{-1}, range: 6.2–10.5 m s^{-1}, N=8). There was no significant difference between the groups regarding flight duration ($F_{1,16}$=0.117, P=0.737) or speed ($F_{1,14}$=0.006, P=0.938).

Question 2: effect of flight on levels of constantly maintained aspects of immune function

We first tested for pre-existing differences in bacterial killing ability by comparing baseline values among the three experimental groups (Fig. 2). No significant difference among groups was detected ($F_{2,23}$=0.490, P=0.619). We did, however, find a significant effect of having performed a flight on bacterial killing ability ($F_{2,18}$=4.295, P=0.030; Fig. 2). A *post hoc* test showed that flown-sick birds had significantly lower bacterial killing ability values than the not-flown birds (P=0.030). The levels in the flown-healthy group were not different from the not-flown group (P=0.085), but tended to be lower. Flown-sick and flown-healthy birds did not differ in killing ability after flight (P=0.887). Flying thus appears to reduce bacterial killing ability, but only significantly so in birds mounting an acute phase response. There were no pre-existing differences in baseline haptoglobin levels among the three experimental groups ($F_{2,19}$=0.247, P=0.783). Flight did not affect haptoglobin levels ($F_{2,20}$=1.029, P=0.376). There was no significant difference among groups in baseline agglutination levels ($F_{2,19}$=0.247, P=0.783), nor were agglutination levels affected by flight ($F_{2,16}$=0.467, P=0.635).

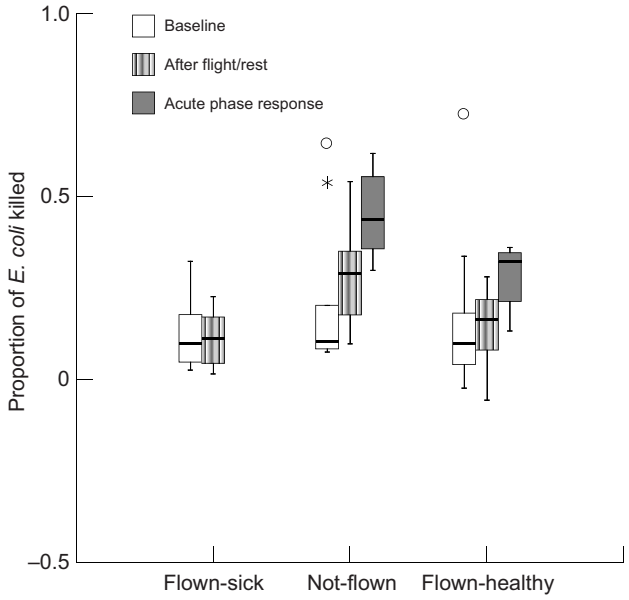

Fig. 2. Effects of an endurance flight and of being flown-sick on bacterial killing ability. Flown-sick birds had significantly lower killing ability after flight/rest than not-flown birds and there was a non-significant trend (P=0.085) of lower killing ability in flown-healthy than in not-flown birds (striped boxes). Flown-healthy birds had significantly lower killing ability while mounting an acute phase response than the not-flown birds (grey boxes). In the not-flown but not the flown-healthy birds, killing ability significantly increased during the acute phase response (white *versus* grey boxes within groups). The box plots show the median (thick horizontal line), 50% range (box), range (whiskers), outliers (asterisk) and extreme outliers (circles).

The same was found for lysis levels: the three groups did not differ significantly in baseline (Kruskal–Wallis: K=0.769, P=0.681) or after-flight (Kruskal–Wallis: K=2.383, P=0.304) lysis levels.

Question 3: effect of flight on acute phase response

We then asked whether performing a flight affects the acute phase response. Note that flown-sick birds are not part of this experiment, as they had already mounted an acute phase response during the flight. While mounting an acute phase response, birds that had been flown on the day prior (flown-healthy) lost a mean of 1.2±1.3 g body mass (N=7), and those that had not flown lost on average 2.0±0.4 g (N=8). The difference in mass change during the acute phase response was not significant between the two groups ($F_{1,13}$=2.99, P=0.107). However, birds that had flown increased their body temperature during the acute phase response on average by 1.9±1.06°C (N=8), while the not-flown birds decreased their body temperature by 0.3±2.32°C (N=8; Fig. 3). The difference in body temperature change during the acute phase response was significant between the two groups ($F_{1,14}$=5.47, P=0.035).

We also compared killing ability, haptoglobin levels, agglutination and lysis between flown-healthy and not-flown birds as well as within groups before and after the acute phase response. We found that flown-healthy birds had significantly lower killing ability while mounting an acute phase response than not-flown birds ($F_{1,10}$=7.012, P=0.024). In the not-flown group, killing ability significantly increased during the acute phase response (t=–2.763, d.f.=6, P=0.033), while in the flown-healthy group, the difference was not significant (t=–1.395, d.f.=4, P=0.236; Fig. 2). There was no difference in haptoglobin levels while mounting an acute phase

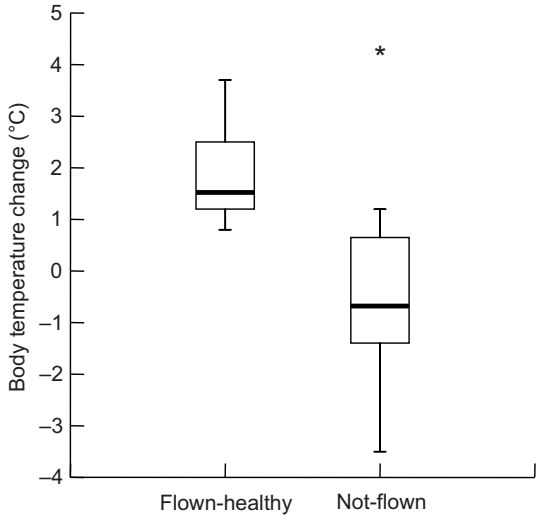

Fig. 3. Western sandpipers that had performed an experimental flight increased their body temperature during the acute phase response, while the not-flown group had a slightly lowered body temperature. The box plots show the median (thick horizontal line), 50% range (box), range (whiskers) and outliers (asterisk).

response between flown-healthy and not-flown birds ($F_{1,12}$=0.012, P=0.915). Haptoglobin levels did not differ significantly between baseline and acute phase response in either the not-flown (t=–0.049, d.f.=3, P=0.964) or in the flown-healthy group (t=–0.605, d.f.=7, P=0.564). Agglutination levels while mounting an acute phase response tended to be lower in flown-healthy than in not-flown birds, but the difference was not statistically significant ($F_{1,12}$=3.843, P=0.074). Agglutination levels did not differ significantly between baseline and acute phase response in not-flown (t=0.415, d.f.=5, P=0.695) or flown-healthy birds (t=–0.493, d.f.=7, P=0.637; Table 1). Finally, lysis levels in flown-healthy and not-flown birds did not differ during the acute phase response (Kruskal–Wallis: K=0.101, P=0.751), nor did lysis levels differ significantly between baseline and acute phase response in not-flown (Kruskal–Wallis: K=0.040, P=0.842) or flown-healthy birds (Kruskal–Wallis: K=0.424, P=0.515; Table 1).

Question 4: relationship between immune function and flight duration

We examined correlations between immune function and flight duration for bacteria killing ability, haptoglobin, agglutination and lysis. We analysed flown-sick and flown-healthy birds separately because flown-sick birds were mounting an acute phase response during flight, and flown-healthy birds after flight. For flown-healthy birds, correlations were tested at baseline, after flight and during the acute phase response. For flown-sick birds, correlations were tested at baseline and after flight. In flown-healthy birds, flight duration was positively correlated with baseline killing ability ($F_{1,7}$=8.257, P=0.024) and very strongly negatively correlated with killing ability during the acute phase on the following day ($F_{1,3}$=629.15, P<0.001; Fig. 4). In flown-sick birds, flight duration was positively correlated with killing ability after flight ($F_{1,6}$=13.109, P=0.011; Table 1). Finally, in flown-healthy birds, flight duration was positively correlated with baseline haptoglobin levels ($F_{1,7}$=13.599, P=0.008; Fig. 4). All other correlations did not reach statistical significance (P>0.27).

DISCUSSION

We used western sandpipers to experimentally test whether flight performance is affected when long-distance migrants are mounting an immune response and whether migrants maintain immune defences during a flight in a wind tunnel. We measured five indicators of innate immunity in birds flying in a wind tunnel without mounting an immune response (flown-healthy), birds flying while mounting an acute phase response (flown-sick) and a non-flying control group (not-flown). Our findings suggest that flight performance is not diminished by prior immune challenge, that flight while mounting an acute phase response negatively affects other aspects of immune function, and that the strength of some aspects of the immune function positively correlates with flight capacity. We discuss these findings below.

Flight performance is not affected by prior immune challenge

Contrary to our prediction that flight performance would be diminished when long-distance migrants are mounting an immune response, flown-sick birds did not fly at lower speed or for less time than flown-healthy birds, indicating that mounting an acute phase response to simulated infection did not hamper an individual's ability to fly for up to 3 h. One could argue that the LPS dosage we used was too low to simulate sickness, but we think this is unlikely because the dosage elicited an acute phase response in Japanese quail, *Coturnix coturnix japonica*, and red knots (Koutsos and Klasing, 2001; Buehler et al., 2009). More importantly, it increased body temperature of western sandpipers in this experiment. It thus seems that western sandpipers are able to maintain their normal flight performance while being sick.

Table 1. Immune indicators in western sandpipers by timing of sample

Timing of sample	Experimental group	Bacterial killing (proportion killed)	Haptoglobin (mg ml^{-1})	Lysis (–log2 titer)	Agglutination (–log2 titer)
Baseline	Not-flown	0.22±0.22 (9)	0.76±0.26 (6)	4.8±0.8 (9)	10.2±2.3 (9)
	Flown-healthy	0.18±0.23 (9)	0.64±0.47 (9)	4.7±1.7 (9)	9.3±3.3 (9)
	Flown-sick	0.13±0.10 (8)	0.76±0.43 (7)	4.1±1.7 (9)	10.3±2.0 (9)
After flight/rest	Not-flown	0.29±0.16 (6)	0.93±0.44 (8)	3.8±0.4 (6)	9.8±2.9 (6)
	Flown-healthy	0.14±0.12 (7)	0.91±0.48 (6)	3.4±0.8 (6)	9.7±3.3 (6)
	Flown-sick	0.11±0.08 (8)	1.19±0.44 (9)	4.0±0.6 (7)	7.6±3.6 (7)
Acute phase response	Not-flown	0.45±0.13 (7)	0.85±0.38 (6)	5.2±1.2 (6)	9.9±2.6 (6)
	Flown-healthy	0.28±0.10 (5)	0.83±0.36 (8)	4.1±0.9 (8)	10.4±1.8 (8)
	Flown-sick	n/a	n/a	n/a	n/a

Data are means ± s.d. (N). Values for bacterial killing are the proportion of *Escherichia coli* colonies killed by the plasma. Values for haptoglobin indicate plasma concentrations. Agglutination and lysis levels indicate the step in the serial dilution of the plasma at which the reaction stopped. For timing of sample, please refer to Fig. 1. Differences in sample sizes among assays were due to limited plasma volume.

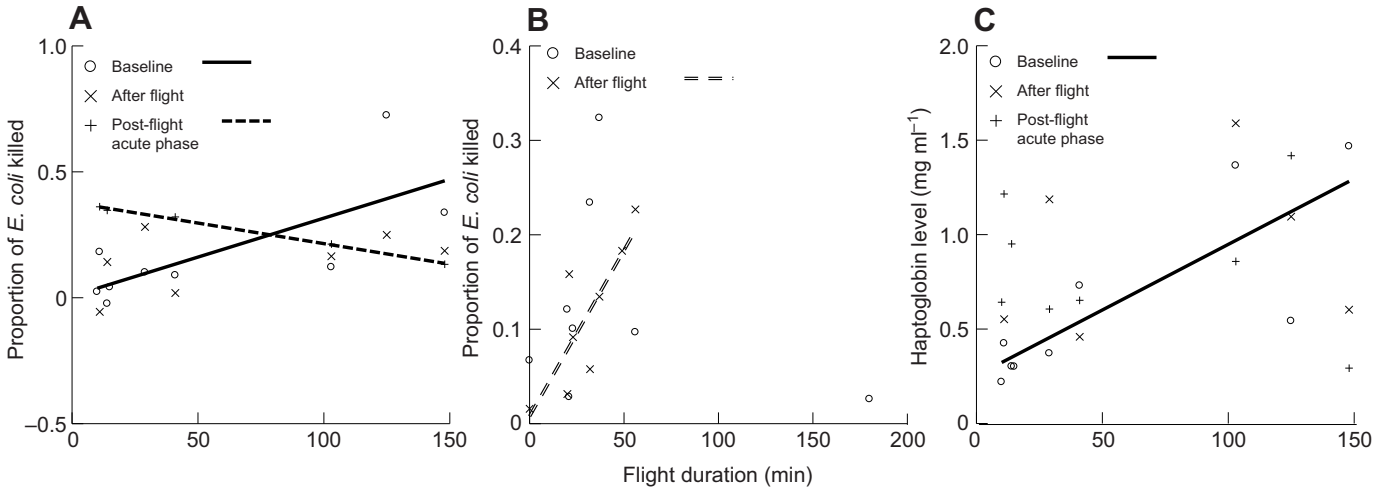

Fig. 4. Correlation of flight duration with killing ability (A,B) and haptoglobin levels (C) in flown-healthy (A,C) and flown-sick birds (B). Regression lines are shown for significant correlations only. (A) In flown-healthy birds, flight duration was positively correlated with baseline killing ability, and negatively correlated with killing ability during the acute phase response (post-flight). Note that the × symbols are masked by the regression line. No correlation was detected with after-flight killing ability. (B) In birds mounting an acute phase response during flight (flown-sick), flight duration was significantly correlated with killing ability after flight, but not with baseline killing ability. Note that the scale on the *y*-axis in A and B differs. (C) In flown-healthy birds, flight duration was correlated with baseline haptoglobin levels, but not with levels after flight or during the acute phase.

Our finding that flight performance in western sandpipers remains unhampered when mounting an acute phase response is consistent with a study that showed that experimental inoculation with an avian influenza virus (H4N6) did not delay the movement or reduce the apparent fitness of free-living Bewick's swans (Hoye, 2011). Swans forced to mount an immune response against a novel antigen (phytohemagglutin) were likewise not hampered in their migratory movements in comparison to sham-treated (PBS) birds. The limited empirical evidence to date suggests that migrating birds do not appear to reduce their flight performance as a consequence of mounting an immune response. Immune function, in contrast, has been shown to diminish after strenuous flights in western sandpipers, European starlings and red knots (Buehler et al., 2010; Nebel et al., 2012).

Flight affects bacteria killing ability in birds mounting an acute phase response

We found a significant effect of having performed a flight on bacterial killing ability. While this effect was only statistically significant in birds that were mounting an acute phase response during the flight, the near-significant (P=0.085) difference between flown-healthy and not-flown birds does suggest a trade-off in which western sandpipers prioritize flight over immune function, particularly when taking the small sample sizes into account. Furthermore, we were able to detect this effect even though we used plasma samples that had been frozen for 4 months. Keeping samples frozen for more than 20 days reduces killing ability by more than 80% (Liebl and Martin, 2009). This suggests that the effect of flight on killing ability is very strong, and that the non-significant difference between flown-healthy and not-flown birds would likely be highly significant if the samples had been processed immediately.

There was no effect of flight on haptoglobin, lysis or agglutination levels. These results are consistent with the findings of a recent study on homing pigeons, *Columba livia*, which did not find a difference in plasma haptoglobin concentration or levels of agglutination between birds that raced and those that did not (Matson et al., 2012).

Effect of flight on the acute phase response and levels of constantly maintained aspects of immune function in birds mounting an acute phase response

The magnitude and the duration of the change in body temperature in response to LPS injection can vary with a number of factors, such as age (Fraifeld and Kaplanski, 1998; Nebel et al., 2013), dose (Maloney and Gray, 1998), population (Adelman et al., 2010b; Adelman et al., 2010a) and season (Owen-Ashley and Wingfield, 2007). Here, we demonstrated a significant effect of flight on body temperature change following LPS challenge. Birds that had performed a flight prior to mounting an acute phase response (flown-healthy) increased their body temperature by 1.9°C, while birds not flying during the acute phase response (not-flown) decreased their body temperature by 0.3±2.32°C (N=8; Fig. 2). The decrease in body temperature in the not-flown birds was somewhat surprising because birds that are exposed to LPS usually develop a fever (see Owen-Ashley and Wingfield, 2007) and our LPS dosage trial showed a significant increase in body temperature in western sandpipers following LPS injection. Nevertheless, hypothermia can also occur (Owen-Ashley et al., 2006).

We also assessed whether performing a flight affected levels of constantly maintained aspects of innate immune function during the acute phase response. After flight, flown-healthy birds had significantly lower bacterial killing ability while mounting an acute phase response than not-flown birds. Agglutination levels showed the same trend. These results provide further evidence for reduced levels of some aspects of innate immunity after a flight. Interestingly, in the not-flown but not the flown-healthy birds, killing ability significantly increased during the acute phase response, which is consistent with a finding in red knots (Buehler et al., 2009). A recent meta-analysis estimates that energy consumption during an immune response rises by 5–15% (Hasselquist and Nilsson, 2012). Our results suggest that flown-sick birds, which were mounting an acute phase response during the flight, had fewer resources left to maintain other aspects of immune function after having performed a flight, providing further evidence that western sandpipers prioritize flight over immune function.

Bacteria killing and haptoglobin levels may predict flight duration

We were also interested in the relationship between immune function and flight duration, in particular whether flight duration could be predicted by immune indicators. In flown-healthy birds, flight duration was positively correlated with baseline killing ability and baseline haptoglobin levels. It is possible that these two immune indicators are indicative of the overall quality of a bird. Indeed, indices of immune function are often assumed to reflect fundamental attributes of individuals (Hill, 2011), and Buehler et al. (Buehler et al., 2008) showed that in another shorebird species, the red knot, measures of baseline killing ability against *E. coli* were significantly repeatable within individuals.

Conclusions

To summarize, we showed that in western sandpipers mounting an acute phase response did not hamper an individual's ability to perform flights in a wind tunnel lasting up to 3 h. Many shorebirds are capable of flying long distances non-stop; for example, bar-tailed godwits, *Limosa lapponica*, cross the Pacific Ocean in a single flight (Gill et al., 2005). Western sandpipers typically perform a series of flights between breeding and non-breeding areas (Wilson, 1994). However, radio telemetry data indicate that migrating western sandpipers regularly fly for 12 or more hours, and are capable of travelling as far as 3250 km in less than 42 h, which requires a multi-day non-stop flight (Iverson et al., 1996). The 3 h flight treatment used in our study allowed birds to reach steady state exercise and be physiologically challenged, but likely mimicked only the first part of a migratory flight between stop-overs. Despite the short flight-duration, bacterial killing ability was reduced after flight, though this result was statistically significant only in birds mounting an acute phase response.

This is, to our knowledge, the first study that assesses performance trade-offs between flight and both constantly maintained and induced innate immunity. These findings have implications for our understanding of the transmission of avian-borne diseases such as avian influenza, as they suggest that birds are still able to migrate while mounting an immune response against an infection. This is particularly relevant for mathematical models that try to predict the spread of avian influenza, as they usually need to incorporate the likelihood of migrating upon infection. Future experiments could vary the dosage of LPS injected and the time between injection and flight, use different types of antigens, and aim for longer wind tunnel flights or use telemetry to follow injected birds in the field.

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AUTHOR CONTRIBUTIONS

S.N., C.G.G. and D.M.B. conceived the study. S.N. and A.M. carried out the experiments. S.N. and D.M.B. wrote the paper, with input from C.G.G. and A.M.

COMPETING INTERESTS

No competing interests declared.

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