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Long flights do not influence immune responses of a long-distance migrant bird: a wind-tunnel experiment

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Summary

Heavy physical work can result in physiological stress and suppressed immune function. Accordingly, long-distance migrant birds that fly for thousands of km within days can be expected to show immunosuppression, and hence be more vulnerable to infections *en route*. The red knot *Calidris canutus* Linnaeus is a long-distance migrant shorebird. We flew red knots the equivalent of 1500 km over 6 days in a wind tunnel. The humoral and cell-mediated immune responses of the flyers were compared to those of non-flying controls. Humoral immunity was measured as antibody production against injected diphtheria and tetanus antigens, and cell-mediated response as phytohemagglutinin-induced wing-web swelling. Blood corticosterone levels, which may modulate immune function, were measured in parallel. The long flights had no detectable effects on humoral or cell-mediated immune responses, or on corticosterone levels. Thus, flight performance *per se* may not be particularly stressful or immunosuppressive in red knots. Some birds assigned as flyers refused to fly for extended periods. Before flights started, these non-flyers had significantly lower antibody responses against tetanus than the birds that carried out the full flight program. This suggests that only birds in good physical condition may be willing to take on heavy exercise. We conclude that these long-distance migrants appear well adapted to the work load induced by long flights, enabling them to cope with long flight distances without increased stress levels and suppression of immunity. Whether this also applies in the wild, where the migrating birds may face adverse weather and food conditions, remains to be investigated.

Key words: stress-induced immunosuppression, red knot, corticosterone, immunocompetence.

Introduction

Strenuous exercise in humans and mice can result in suppressed immune function (Hoffmann-Goetz and Pedersen, 1994; Nieman and Nehlsen-Cannarella, 1994). In birds, immunosupression has been found in individuals exposed to experimentally increased workload during nestling provisioning (Deerenberg et al., 1997; Moreno et al., 1999; Hasselquist et al., 2001). These studies suggest that activities imposing a high metabolic rate suppress the immune defense (Råberg et al., 1998). Such exercise-induced immunosuppression may be adaptive, for example to save energy (Sheldon and Verhulst, 1996) or to avoid autoimmune reactions (Råberg et al., 1998).

Bird flight is generally the hardest type of work carried out by animals, with a power output corresponding to between 4 and 60× the basal metabolic rate BMR (Pennycuick, 1989). This can be compared to the daily levels of power output in reproducing birds of 3–5× BMR (Drent and Daan, 1980; Bryant and Tatner, 1991). In the most extreme cases of long-distance migratory birds, the very high work levels may be sustained for several days, during which the birds may lose up to half their pre-flight body mass (Battley et al., 2001; Gill, Jr et al., 2005). Hence, long-distance migrant birds that may fly for many tens of hours without rest may well show immunosuppression, with an increased risk of contracting infection *en route.*

The immune system may be regulated by stress hormones (Sapolsky, 1992; Golding and Flower, 1997). In birds, high levels of the stress hormone corticosterone have been found to correlate with immunosuppression (Evans et al., 2000; Owen-Ashley et al., 2004). In long-distance migrating birds, blood corticosterone levels are generally variable over the migratory

cycle, with elevated levels just before take-off (Piersma et al., 2000; Landys-Ciannelli et al., 2002) and sometimes just after landing (Landys-Ciannelli et al., 2002) (but see Gwinner et al., 1992; Jenni et al., 2000). Elevated corticosterone levels have also been found in connection with migratory fat deposition (Landys-Ciannelli et al., 2003; Long and Holberton, 2004; Dallmann et al., 2004) (but see Piersma et al., 2000; Landys-Cianelli et al., 2002). Given that corticosterone levels at times are high during migration, this could be the mechanism mediating immunosuppression.

We investigated how the immune responses and stress hormone levels were affected when long-distance migrating red knots *Calidris canutus* Linnaeus were flown repeatedly for long periods in a wind tunnel. Red knots are well-adapted flyers that routinely make very long uninterrupted flights between their High Arctic breeding areas and the coastal wintering areas (Piersma and Davidson, 1992; Piersma et al., 2005). Moreover, these birds adjust easily to conditions in the wind tunnel and have repeatedly flown for long periods of time, seemingly undisturbed by the artificial circumstances (Lindström et al., 2000; Kvist et al., 2001; Jenni-Eiermann et al., 2002).

Given that strenuous work generally suppresses immune function, we expected birds exposed to the long-flight treatment to have suppressed immune responses compared with non-flying controls. In parallel, to investigate the potentially modulating role of stress hormones on immune responses, we investigated blood corticosterone levels.

Materials and methods

Overall experimental protocol

Twenty-three juvenile (first calendar year) red knots *Calidris canutus* L. were trapped at a night-time roost site, Richel, in the western Dutch Wadden Sea (53°16′N, 5°08′E), on 29 and 30 August 2000. After capture the birds were kept at the NIOZ experimental shorebird facility on Texel, The Netherlands (53°05′N, 4°75′E).

Because only a few birds can be flown in the wind tunnel at the same time, we divided the birds into two batches. When the first batch (Batch 1, 10 birds) was in Lund for the experiments, the other group (Batch 2, 10 birds) remained in The Netherlands (Table 1). Hence, Batch 2 had a longer period between trapping and experiment than Batch 1, and we therefore entered Batch as a variable in the statistical analyses. Otherwise the two batches had identical treatments. The experiments in Lund were done on 11–30 September 2000 (Batch 1) and 27 October–16 November 2000 (Batch 2).

After sampling blood for baseline immune values, a first diphtheria–tetanus (DT) injection was given to all birds in The Netherlands on 31 August. After 14 days, a blood sample was collected to measure the primary immune response. After arrival at Lund and a day of rest, the birds were divided into a Flyer and a Control group (see "Flight Training" below). Over the next 6 days the Flyers were trained to fly in the wind tunnel in daily sessions (see "Flight Training" below), whereas the Control birds stayed in the cage. The day after Flight Training stopped all birds got the second DT injection, and the next day the Flight Treatment session started (see below). The basic idea was to fly each bird for as long as possible during 1 week. The day after the Flight Treatment week a third blood sample was taken to measure secondary immune responses. Later the same day the birds were injected with phytohemagglutinin (PHA) in the wing web and after 24 h we measured the PHA-induced swelling as an estimate of cell-mediated immune responses. These two immune response measures were used to investigate the effect of strenuous flight exercise on humoral and cell-mediated immune function.

Assigning birds to experimental treatments

The ten birds of Batch 1 were randomly selected from 19 of the 23 birds. Four of the 23 birds had some initial problems adjusting to the aviaries and were therefore excluded from the first draft, but these four birds soon recovered. When selecting the birds for Batch 2 in late October (out of the remaining 13 birds), we excluded the three heaviest birds (>220 g), because in our experience such excessively fat birds are less prone to fly. All birds were brought back to The Netherlands directly after the experiments ended in Lund.

We assigned six Flyers and four Controls within each Batch. This uneven design was because we expected that some birds would not adjust to flying in the wind tunnel (see more below about Failed Flyers). We first paired the birds in order of mass and randomly assigned one bird in each pair to Flyers and the other to Controls. The two birds with body mass in the middle range were both assigned Flyers.

Within each Batch, we then randomly selected three Flyers and two Controls to be sampled in the morning and afternoon, respectively, because only five birds could be sampled for a baseline corticosterone level within the required 3 min from catching.

Housing conditions

In The Netherlands the birds were held in outdoor cages (4 m×1.5 m×2 m). The birds had access to a small saltwater mudflat, with saltwater running over the adjacent concrete floor. A basin with running freshwater could be used for bathing and drinking. Aviaries were cleaned once a week, during which the condition of the birds was checked.

In Lund the birds were kept in an aviary (3 m×1.5 m×2 m) in the wind tunnel building, and kept on a 12 h:12 h L:D cycle. The cage floor was covered with a plastic carpet ('artificial turf') soaked with salt water. Freshwater for bathing was supplied in a small basin. The aviary was cleaned and new water was supplied on a daily basis. Temperature in the wind tunnel hall changed from around +20°C in early September to +10°C in November.

At both sites the birds were fed on commercial trout pellets *ad libitum* and had continuous access to freshwater. The size of the aviaries did not allow fast forward flight, hence the only real flight exercise the birds got was obtained in the wind tunnel.

Upon arrival in Lund the birds were left undisturbed for 1

Table 1. *Summary of the terms used to describe the experimental protocol*

Term	Explanation
Experimental Treatment	All birds (*N*=20) were initially assigned either as Flyers or Controls. One assigned Flyer was excluded due to illness, leaving a total of 19 birds.
Flyers	Birds assigned to fly in the wind tunnel and successfully flew the equivalent of about 1500 km during 1 week (*N*=6).
Controls	Birds assigned not to fly in the wind tunnel (*N*=8).
Failed Flyers	Birds originally assigned to be Flyers, but did not successfully complete the Flight Treatment (*N*=5).
Flight Training	1 week of training in the wind tunnel of the birds assigned as Flyers.
Flight Treatment	The 1 week of long flights in the wind tunnel following the Secondary injection.
Batch	Batch 1 and 2 (*N*=10 and *N*=9) started their Experimental Treatment 11 and 57 days after capture, respectively.
Immune Challenges	First and second injections of diphtheria–tetanus (DT) antigens in the breast muscle; phytohaemagglutinin (PHA) injection in the wing web.
Cell-mediated response	Measured as skin-swelling response 24 h after injection of PHA in the wing web.
Humoral responses	Measured as antigen-specific antibody levels in blood before or a number of days after a DT injection in breast muscle.
Baseline response	Antibody levels measured from blood sample taken just before the first DT injection.
Primary response	Antibody levels measured from blood sample taken 14 days after the first DT injection.
Secondary response	Antibody levels measured from blood sample taken 8 days after the second DT injection.
Corticosterone levels	Corticosterone levels in blood sampled at certain occasions during the experimental protocol.
PreTreat	Corticosterone levels 1 day after the birds arrived to the wind tunnel.
PostTreat	Corticosterone levels the day after the Flight Treatment ended. Sampled at the same time as the Secondary response.
Baseline	Corticosterone levels within 3 min from catching a bird.
Stress-induced	Corticosterone levels ca. 30 min from catching a bird.

day before activities started. It should be noticed that juvenile red knots rapidly get almost hand-tame when handled daily, and the birds would only be visibly stressed when a person went into the aviary to capture them.

Body mass

All birds in Batch 1, independent of Experimental Treatment, had very similar body mass development. They arrived in Lund weighing on average 124 g and gradually increased in body mass to around 140 g. Batch 2 was a more heterogeneous group. Two control birds kept a constant mass of 130 g from arrival to departure, while two other controls decreased their mass from 190 g to 105 g at the end of the experiment. Then both started to feed and 1 month later, while back in The Netherlands, they both weighed around 130 g. There were no signs during the stay in Lund that they were unhealthy. Voluntary long fasts are a natural feature of fat captive red knots (Piersma and Poot, 1993; Piersma, 2002). The assigned Flyers varied on arrival between 110 g and 190 g. The heaviest birds all decreased in mass over the first 10 days, levelling out at around 130 g. From then on all assigned Flyers maintained a stable mass.

Immunization and blood sampling

The birds were injected with 120 μl of DT vaccine (tetanus toxoid (7.5 Lf) and diphtheria toxoid (30 Lf) mixed with the adjuvant aluminium phosphate (5 mg ml^{-1}; SBL Vaccine, Stockholm, Sweden) for the first (primary) injection, and 100 μl of DT vaccine for the second (secondary) injection. The injections were given intramuscularly in the breast muscle.

Blood samples were taken just before the first injection, 14 days after the first injection (at the presumed peak of the primary immune response) and 8 days after the second injection (at the presumed peak of the secondary immune response) (Hasselquist et al., 1999). All blood samples were taken by puncturing the brachial vein and collecting a total of 75–125 μl of blood in heparinized microcapillaries.

Blood samples for corticosterone analyses were collected at the same time as the blood for humoral immune response measurements. When measuring baseline levels of corticosterone, the birds should not be stressed by external cues (such as people) before sampling. With our red knots it was impossible to approach the cage without the birds noticing us; however, the birds were relaxed in our presence and used to people talking outside the cage. Before we entered the cage we therefore spent 30–45 min in the wind-tunnel building, and 10 min before the blood sampling we were talking quietly near the cage. Then one person entered the cage to rapidly trap the five focal birds, which all were sampled within 3 min from entering the cage. From these pre-treatment samples, we measured what we call ‘baseline’ corticosterone levels in birds that to us appeared calm and un-stressed before we entered the cage to catch them. After the baseline sample was taken the birds were kept together in a small darkened cage and a second blood sample was taken ca. 30 min after catching (i.e. ‘stress-induced’ samples). The baseline values were always

considerably lower than the stress-induced values (S.J.-E., unpublished data), suggesting that the baseline values were not taken from stressed birds. This was also true for all Control birds that showed a normal steep increase in corticosterone levels when exposed to handling stress (i.e. much higher corticosterone values in stress-induced as compared with baseline samples).

Cell-mediated and humoral immune response

We investigated the capacity of the immune system to react to antigens by measuring two central aspects of acquired immunity, cell-mediated and humoral immunity. These two measures are both dependent on activation of the innate immune system (e.g. macrophages, complement system, inflammation), hence providing integrated measures of a cascade of immune function.

Measuring cell-mediated immune responses

We measured T-cell mediated immune responses as the size of the wing-web swelling caused by an injection with PHA. Smits et al. (Smits et al., 1999) convincingly showed that a sham injection in the wing web does not result in any swelling at all and as a consequence the standard procedure for the wing web swelling test in immunoecology studies is nowadays to exclude the sham injection. Hence, we injected 0.2 mg PHA (Cat. L-8754, Sigma) dissolved in 20 μl of sterile PBS intradermally in the right wing web (patagium) of the birds, leaving the left wing un-injected (Smits et al., 1999). We measured the thickness of the patagium to the nearest 0.01 mm with a micrometer immediately before and 24 h after antigen injection. All measurements were taken by one person blind to the treatment (D.H.), while another person held the bird with the right wing out in a normal position. The patagium feathers at the area of injection was soaked with 70% ethanol and moved aside to leave bare skin at the swelling, and the micrometer was tightened just until the skin began twisting (Ohlsson et al., 2002). We used the mean of three measurements as the measure of thickness, and used the difference in thickness caused by the PHA injection (after – before) as our measure of cell-mediated immune responses.

It is worth pointing out that the PHA-induced skin-swelling test not only measures the T-cell mediated activity of acquired immunity, but also that important aspects of innate immunity, such as macrophages and inflammation response, contribute to the size of the skin-swelling response (Martin et al., 2006a). Hence, the skin-swelling test provides an integrated measure of important aspects of innate and cell-mediated immunity.

Measuring humoral immune responses

We used a modified quantitative enzyme linked immunosorbent assay (ELISA) to measure blood concentration of tetanus- and diphtheria-specific antibodies (Hasselquist et al., 1999; Hasselquist et al., 2001) [for details of protocol, see Mendes et al. (Mendes et al., 2006)]. Plasma samples were diluted 1:1200, 1:1800, 1:2400 and 1:3600. For each individual, we subtracted the pre-injection antibody titer from the primary and secondary antibody titers, respectively (as we considered the pre-injection titer as unspecific binding to the antigens), to obtain measures of antibody responses specific to the respective antigens. The primary and secondary antibody titers were then $\log_{10}$-transformed before being entered in the statistical analyses.

A duplicate of each sample was run (within-individual variability was 6.3%) and the average of these duplicate sample values was used in all subsequent analyses. As a reference on all plates, we made a dilution series on a sample from a previously challenged red knot with high secondary humoral responses (starting at 1:600 and diluting 1:2 to 1:9600). We corrected for inter-plate variability by reducing all data to the same reference plate, using the red knot reference sample. Based on the standard samples, the overall inter-plate variability (within and between batches) was 11.7%. We used the antibody titer values obtained from the 1:1200 dilutions for diphtheria toxoid and from the 1:1800 dilutions for tetanus toxoid. These dilutions were chosen because pre-injection antibody titer values were low whereas post-injection values were still relatively high (according to standard procedures in the Hasselquist ELISA lab).

Flights

A detailed description of the Lund wind tunnel is given elsewhere (Pennycuick et al., 1997), also more information about the flight performance of red knots (Lindström et al., 2000; Kvist et al., 2001; Jenni-Eiermann et al., 2002). In short, one or two birds fly in an open test section that is 1.20 m wide, 1 m high and 2 m long, with transparent walls and a 50 cm wide opening that allows a person to work closely with the birds in the air current. The earlier studies showed that at wind speeds around 15 m s^{-1} the red knots fly most comfortably. Our birds were therefore trained and flown at this speed. The air in the tunnel was cooled to +10–14°C during the Flight Treatment, which is below the critical temperature when dehydration in flying knots may occur in the wind tunnel (Kvist, 2001).

Flight Training

During each of the Flight Training days, each bird spent up to 1 h,30 min in the tunnel. Training flights varied in length between a few seconds and 40 min. Total training flight time was on average 2 h,25 min for the 12 Flyers, and varied between 13 min and 3 h,23 min.

Flight Treatment and Failed Flyers

In total 12 birds were assigned Flyers and included in the Flight Treatment. Six of them (three from each batch) managed to fly successfully for the full week, in total between 26 h and 28 h,45 min. This is equivalent to 1400–1550 km in still air. The successful flyers were flown in sessions of 2, 4 or 10 h. The birds were flown either alone or in pairs. Pair flights were initiated to maximize the total flight time achieved within the week. On four occasions we had pair flights lasting 10 full hours.

A total of six birds assigned as Flyers did not get through

the planned flight programme for Flyers, and we call these birds Failed Flyers. The reason for their failure differed between birds. Four of the birds were generally nervous when in the wind tunnel and although some of them did fly the odd hour, they would eventually land and refuse more flying. A fifth bird that had flown successfully 2 h and 10 h crashed at the start of the third flight and then refused to fly. The sixth bird got seriously ill and was excluded from the experiment altogether.

Corticosterone analyses

Blood samples taken for corticosterone analysis were immediately centrifuged for 7 min at 850 ***g*** and stored at –30°C within 1 h. The samples were sent frozen to Switzerland on 14 May 2002 where they were analysed for corticosterone.

Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro and Lasley, 1988). Corticosterone in 10 μl of plasma (diluted 1:20 in distilled water) was extracted with 4 ml dichlormethane, re-dissolved in phosphate buffer and analysed in triplicate. A sheep-anti-corticosterone polyclonal antibody was used at a final dilution of 1:8000 (Chemicon Int., Temecula, CA, USA). The concentration of corticosterone in plasma samples was calculated from a standard curve run in duplicate on each plate. A plasma pool from chickens (*Gallus domesticus* Linnaeus) was used as an internal control on each plate. The mean intra-assay coefficient of variation was 7.2% and the mean inter-assay coefficient of variation 18.9%. The assays were performed in duplicate in June 2002.

Statistics

We used parametric statistics throughout. For ANOVAs we always first tested for significant interaction terms. Non-significant interaction terms as well as non-significant factors were removed from the final model. Using repeated-measures ANOVA we tested whether, within individuals, PostTreat values differed from PreTreat values (for immune responses and corticosterone levels, respectively), and if any such differences depended on whether the birds had flown for long periods or not. ELISA read-outs (antibody titers) were $\log_{10}$-transformed after adding 1 to all values, before use in the statistical analyses. All analyses were carried out using SPSS 11.0 (SPSS Inc.).

Results

Body mass

There were no significant differences in body mass between Experimental Treatment groups (Flyers, Controls and Failed Flyers) at either arrival to Lund, at the time for the second DT injection, or after the Flight Treatment (one-way ANOVAs, $P>0.4$ in all cases). Thus, the body mass development of the birds when in Lund was on average similar within the three Experimental Treatment groups of birds. The variation in mass at PostTreat was largely explained by the mass at second injection (two-way ANOVA, $F_{1,15}=15.3$, $P=0.001$), with no simultaneous effect of Experimental Treatment ($F_{2,15}=1.4$, $P=0.29$). That is, whether the birds flew or not during the Flight Treatment week did not influence the body mass development over the same period.

Correlations between immune measures

The birds' Primary and Secondary responses showed significant positive relationships for tetanus (Pearson correlation: $r_{18}=0.574$, $P=0.010$) as well as diphtheria (Pearson correlation: $r_{18}=0.470$, $P=0.042$). That is, birds with a relatively high Primary response against a given antigen also had a relatively high Secondary response to this antigen, and *vice versa*. The birds' responses against the two antigens also showed positive relationships, albeit non-significant, for the Primary antibody response (Pearson correlation: $r_{18}=0.395$, $P=0.094$) as well as the Secondary antibody response (Pearson correlation: $r_{18}=0.346$, $P=0.15$). That is, a bird with a high Primary response against tetanus also tended to have a high Primary response to diphtheria, and there was a similar tendency, albeit weaker, for the Secondary responses. Thus, the ELISA method we used to measure antibody titers provided rather consistent results for within-individual and between-antigen analyses.

At PostTreat there was no significant correlation between the PHA-induced wing web swelling and antibody production against tetanus (Pearson correlation, $r=–0.053$, $P=0.84$) and a tendency for a negative correlation between PHA-induced wing web swelling and the anti-diphtheria response (Pearson correlation, $r=–0.37$, $P=0.13$). Thus, the cell-mediated and humoral immune responses were not positively correlated.

Immune responses of experimental groups before flight

Before the Flight Treatment started, the three treatment groups differed in their primary antibody response against tetanus (two-way ANOVA, $F_{2,16}=4.48$, $P=0.029$, no effect of batch) but not against diphtheria (two-way ANOVA, $F_{2,16}=0.26$, $P=0.78$, no effect of batch). For tetanus, it was the primary response of the group that later would become Failed Flyers that was significantly lower than for the group that later would become Flyers (Tukey's HSD, $P=0.027$), whereas there was no difference between Flyers and Controls (Tukey's HSD, $P=0.70$; Fig. 1). Thus, before the flight treatment started, the birds in the Control and the Flight groups were similar in their immune responses, whereas the birds that became the Failed Flyers had a significantly lower response to the tetanus antigen.

Effects of long flights on immune responses

The Secondary antibody response was significantly higher than the Primary response (repeated measures ANOVA; for tetanus, $F_{1,18}=54.3$, $P<0.001$; for diphtheria, $F_{1,18}=14.8$, $P=0.001$), which is typical for humoral immune responses of vertebrates. Most importantly, there was no significant interaction term between Experimental Treatment and the change in antibody titers between the primary and the secondary response, neither for tetanus nor diphtheria (repeated-measures ANOVA; for tetanus, interaction term

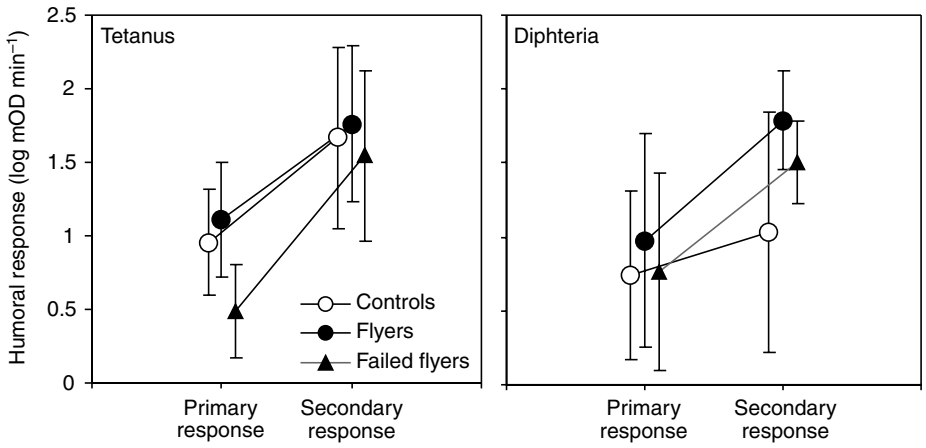

Fig. 1. The Primary (pre-treatment) and Secondary (post-treatment) humoral immune responses (means ± s.d.) to tetanus and diphtheria antigens in red knots subjected to different Experimental Treatments. The treatment was almost identical for all birds up to the Primary response (see text). The week before the Secondary response was measured, Flyers (*N*=6) flew the equivalent of 1500 km in a wind tunnel. Failed Flyers (*N*=5) were birds originally assigned as Flyers that refused to fly for long periods in the wind tunnel. Controls (*N*=8) did not fly in the wind tunnel. The long flights had no detectable effect on the humoral immune response of the birds. Humoral immune responses were measured as the antigen-specific antibody response using a kinetic ELISA. A higher antibody titer against a specific antigen was detected as a faster colour change over time [measured in milli Optical Densities (mOD) min^{-1}], which is equivalent to more antibodies specifically bound to the antigen.

$F_{2,16}$=1.27, P=0.31; for diphtheria, interaction term $F_{2,16}$=1.35, P=0.29, Fig. 1). This suggests that flying approximately 1500 km over 6 days did not affect humoral immune responses in red knots. If we make this analysis comparing only Flyers and Controls, that is, excluding Failed Flyers, the results are essentially the same (interaction term for tetanus: $F_{1,12}$=0.07, P=0.79, interaction term for diphtheria: $F_{1,12}$=2.51, P=0.14). Note that for the diphtheria analysis the weak trend was actually in the opposite direction to the one predicted. If anything, Flyers had a higher increase in their diphtheria titers over the Flight Treatment period than the Controls.

We found no difference in wing web swelling caused by a PHA injection between birds of different Experimental Treatments (two-way ANOVA, $F_{2,14}$=0.76, P=0.48; Fig. 2), but there was a significant effect of Batch ($F_{1,14}$=5.0, P=0.043; birds of Batch 1 had larger swellings than those of Batch 2). When comparing only Flyers and Controls, there was no significant difference in the wing web swelling response to PHA between the Experimental Treatments, but still an effect of Batch (two-way ANOVA, effect of Experimental Treatment, $F_{1,10}$=1.03, P=0.335; effect of Batch, $F_{1,10}$=5.5, P=0.041). Thus, there was no visible effect of long flights on the cell-mediated immune response.

Corticosterone

Baseline corticosterone levels

There were no differences in baseline corticosterone at rest between birds of different Experimental Treatments and Batch, at either PreTreat (two-way ANOVA, effect of Experimental Treatment, $F_{2,15}$=0.67, P=0.53, effect of Batch, $F_{1,15}$=0.01, P=0.94) or PostTreat (two-way ANOVA, effect of Experimental Treatment, $F_{2,15}$=0.29, P=0.75, effect of Batch, $F_{1,15}$=2.47, P=0.14).

We found no significant interaction term between Experimental Treatment and the change in baseline corticosterone between PreTreat and PostTreat (repeated-measures ANOVA; interaction term $F_{2,16}$=1.5, P=0.26, Fig. 3). Further, the changes in individual baseline corticosterone from PreTreat to PostTreat were not significant (repeated-measures ANOVA, $F_{1,18}$=0.77, P=0.20), nor were there any effects of Experimental Treatment or Batch on these differences (repeated-measures ANOVA; effect of Experimental Treatment: $F_{2,15}$=1.38, P=0.28, effect of Batch: $F_{1,15}$=1.79, P=0.20). This indicates that flying approximately 1500 km over 6 days did not affect baseline corticosterone levels in red knots.

Correlation between corticosterone and immune response

Secondary antibody titers of neither tetanus nor diphtheria correlated significantly with baseline corticosterone levels as measured after a week of Flight Treatment (Pearson correlation: tetanus *vs* corticosterone, r_{18}=–0.07, P=0.78; diphtheria *vs* corticosterone, r_{18}=0.09, P=0.71). Including body mass in these analyses did not change any results.

Discussion

The red knot is a long-distance migratory species, and we did not find any evidence that 6 days of intensive flight exposure (equivalent to flying 1500 km) suppressed either humoral or cell-mediated immune responses. This implies that a regular long-distance flyer such as the red knot is physiologically well-adapted to high physical workload in terms of prolonged flight. As a consequence, red knots may be able to perform impressive long flights without being exposed to immunosuppression, which seems highly adaptive given the increased risk of contracting diseases at e.g. stopover sites when *en route* to and from wintering areas (e.g. Piersma, 1997; Waldenström et al., 2002; Mendes et al., 2005). That long flights may not be as physiologically demanding as previously thought in long-distance migrating birds, was also suggested from a study on barnacle geese (*Branta leucopsis* Linnaeus),

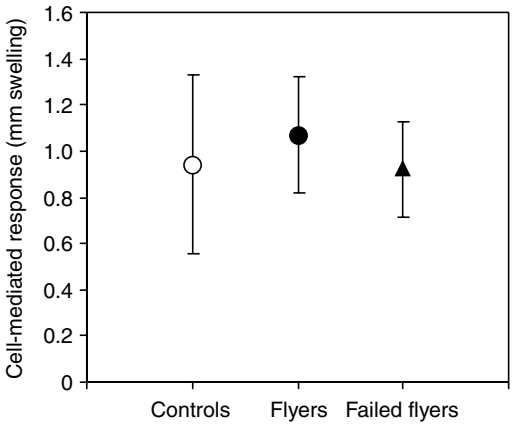

Fig. 2. The cell-mediated immune response (mean ± s.d.), measured as PHA-induced wing-web swelling, in red knots subjected to different Experimental Treatments (as in Fig. 1). The long flights had no detectable effect on the cell-mediated immune response of the birds.

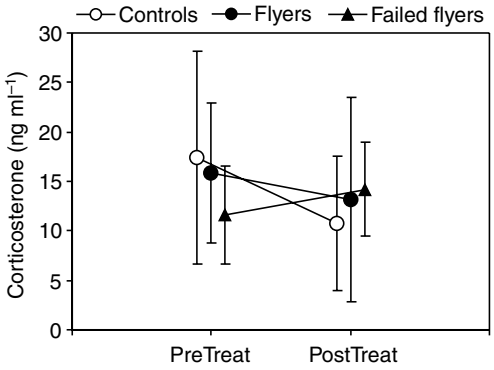

Fig. 3. Baseline corticosterone levels (means ± s.d.) before (PreTreat) and after (PostTreat) the Flight Treatment in red knots. Flyers (*N*=6) flew the equivalent of 1500 km in a wind tunnel. Failed Flyers (*N*=5) were birds originally assigned as Flyers that refused to fly for long periods in the tunnel. Controls (*N*=8) did not fly in the wind tunnel. The long flights had no detectable effects on the corticosterone levels.

which showed lower than expected heart rates for geese migrating in the wild (Butler et al., 1998).

Our method of measuring humoral immune function provided rather consistent measures of antibody levels in red knots. There were positive, albeit rather weak, correlations between the birds' antibody titers against the two antigens (i.e. diphtheria and tetanus) as well as between primary and secondary responses. That our estimates of humoral immune functions were fairly consistent across time and antigens, suggests that we obtained reasonable estimates of the birds' inherent humoral immunity. Hence, contrary to studies on some passerine birds, the antibody titers of individual birds were rather similar for the two antigens and we did not find a stepwise (truncated) relationship between diphtheria and tetanus titers (Westneat et al., 2003; Poston et al., 2005). We found no relationship between cell-mediated (PHA induced wing-web swelling) and humoral (diphtheria and tetanus induced antibody production) immune responses. A lack of relationship between these two components of the acquired immune system has been found in other immunoecology studies of birds (e.g. Owen-Ashley et al., 2004; Martin et al., 2006b). This may be due to the uncoupled or even conflicting interaction at the T-helper cell level between these two arms of the acquired immune system (e.g. Kuby, 1992).

In a number of studies on breeding birds, increased workloads in terms of flying during nestling feeding, have resulted in suppressed humoral (Deerenberg et al., 1997; Nordling et al., 1998; Hasselquist et al., 2001) and cell-mediated (Moreno et al., 1999; Chichon et al., 2001; Saino et al., 2002) immune function. Moreover, this relationship is reciprocal because an antigen injection in breeding birds results in lowered work load (nestling feeding) and negative effects on reproductive success (Ilmonen et al., 2000; Råberg et al., 2000; Bonneaud et al., 2003). Hence, given these earlier studies it was somewhat surprising that we did not find any adverse effect of long flights on any of the physiological measures that we tested. If anything, flying in a wind tunnel may be more energetically demanding than normal migratory flight (Bishop et al., 2002; Bowlin et al., 2005). Hence, even though our sample size is somewhat small (and statistical power thus moderate) due to the limitations of keeping birds in captivity and flying them in the wind tunnel, we had expected large differences between groups, because treatment and control groups were exposed to strikingly different work loads in terms of flight. Instead, we found no differences in acquired immune responses between treatment groups, suggesting that a regular long-distance migrant such as the red knot is physiologically well adapted to flying long distances within a limited time span. This result raises the possibility that birds suffering suppressed immune function when exposed to experimentally increased feeding demands during breeding (e.g. Deerenberg et al., 1997; Chichon et al., 2001; Hasselquist et al., 2001; Saino et al., 2002) have been reacting not only to the higher work load but also to the psychological stress of having unsatisfied young demanding more food. However, in two studies of barn swallows (*Hirundo rustica* Linnaeus) there was no effect of brood size (natural or manipulated) on corticosterone levels in the parents (Saino et al., 2002) (S.J.-E., unpublished observations).

It has been suggested that immunosuppression during heavy work load (e.g. nestling feeding in birds) is an adaptive reallocation of energy as the body cannot run two energy-demanding systems at high activity simultaneously (Sheldon and Verhulst, 1996; Saino et al., 2002). As we did not find any relationship (or even a tendency for a positive trend) between strenuous flight exposure and acquired immune responses in red knots, our study does not support the energy reallocation hypothesis. We cannot exclude that another part of the immune system that we did not measure, e.g. innate immunity, was suppressed to reallocate energy. However, had there been a severe suppression of innate immunity we should have been able to detect this effect because the innate immune response

will influence the magnitude of both the cell-mediated and humoral immune responses.

An interesting finding is that individuals that refused to fly for extended periods of time (i.e. Failed Flyers) already had significantly lower antibody responses against tetanus before flights than birds that did fly 1500 km in a week. We interpret these data as suggesting that only birds in good physiological condition are willing to take on the task of long flights. Hence, there may be hidden costs associated with taking on strenuous exercise (such as long flights) when the body is not prepared. One is the increased risk of immunopathology (Råberg et al., 1998). Such costs may potentially be severe, as suggested by a study on female common eiders (*Somateria mollissima* Linnaeus) where injection with antigens during the strenuous incubation period (when these birds were fasting) resulted in a considerably reduced between-year return rate in birds producing antibodies compared with birds not responding to the antigens (Hanssen et al., 2004).

Baseline corticosterone levels were not affected by experimental treatment. This is in accordance with studies in free-living passerines caught out of their migratory flight (Gwinner et al., 1992) and just after landing (Jenni et al., 2000). These birds also had no elevated corticosterone, except in a few which had completely emaciated breast muscles and no visible fat. The baseline corticosterone values of all birds in our study were very similar to those of wild red knots at their breeding grounds (Reneerkens et al., 2002) and to apparently unstressed red knots that had long been adjusted to living in indoor cages (Piersma et al., 2000). Similarly low values were found in refuelling bar-tailed godwits *Limosa lapponica* Linnaeus in the wild (Landys-Ciannelli et al., 2002), whereas red knots and godwits newly arrived following long flights had substantially higher corticosterone levels (Reneerkens et al., 2002; Landys-Ciannelli et al., 2002). Hence, the Control birds in our study seem to have a normal baseline corticosterone level for wild and captive red knots.

Conclusion

Contrary to our predictions, strenuous exercise in the form of 1500 km of flight during a week did not affect either immune responses or corticosterone levels in red knots flying in a wind tunnel. This suggests that these long-distance migrant birds are well adapted to demanding long flights, and that workload *per se* does not induce immunosuppression. If so, the long migratory flights are not likely to enhance the risk of contracting diseases *en route* between winter and summer quarters. However, our birds were well fed. Whether poor or unpredictable conditions in the wild, such as adverse weather and food constraints on staging sites, may induce stress and immunosuppression remains to be studied.

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