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## RESEARCH ARTICLE

# Intense flight and endotoxin injection elicit similar effects on leukocyte distributions but dissimilar effects on plasma-based immunological indices in pigeons

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## SUMMARY

**Most birds rely on flight for survival. Yet as an energetically taxing and physiologically integrative process, flight has many repercussions. Studying pigeons (*Columba livia*) and employing physiological and immunological indices that are relevant to ecologists working with wild birds, we determined what, if any, acute immune-like responses result from bouts of intense, non-migratory flight. We compared the effects of flight with the effects of a simulated bacterial infection. We also investigated indices in terms of their post-flight changes within individuals and their relationship with flight speed among individuals. Compared to un-flown controls, flown birds exhibited significant elevations in numbers of heterophils relative to numbers of lymphocytes and significant reductions in numbers of eosinophils and monocytes. Furthermore, within-individual changes in concentrations of an acute phase protein were greater in flown birds than in controls. However, none of the flight-affected indices showed any evidence of being related to flight speed. While some of the effects of flight were comparable to the effects of the simulated bacterial infection, other effects were observed only after one of these two physiological challenges. Our study suggests that flight by pigeons yields immune-like responses, and these responses have the potential to complicate the conclusions drawn by ecologists regarding immune function in free-living birds. Still, a better understanding of the repercussions of flight can help clarify the ties between the physiology of exercise and the disease ecology of migration and will ultimately assist in the broader goal of accounting for immunological variation within and among species.**

Key words: birds, Columbiformes, flight, immunology, inflammation, leukocytes.

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## INTRODUCTION

Locomotion is critical to survival in many organisms, but as a broadly integrative physiological function, locomotion has wide-ranging repercussions. Many birds rely on flight to escape from predators, to search for food, to find mates and, more generally, to move among habitats. Yet some forms of flight are strenuous and have costs. These costs range from the relatively short-term energetic costs of a quick escape flight (Nudds and Bryant, 2000) to the considerably longer-term costs associated with the physiological re-organization (Piersma and Lindström, 1997; Gerson and Guglielmo, 2011) and increased energy use (Bundle et al., 2007; Sapir et al., 2010; Pennycuick, 1968; Dawson et al., 1983) that characterize migration. Flight can also lead to tissue damage, for example in the form of increased free-radicals and oxidative stress (Costantini et al., 2008; Larcombe et al., 2010) or direct muscle injury and myofilament degradation (Guglielmo et al., 2001; Bordel and Haase, 2000). Other effects of flight include reductions in haematocrit and in plasma levels of triglyceride and protein and increases in plasma levels of free fatty acid, uric acid, urea and creatine (Bordel and Haase, 1993; Bordel and Haase, 2000).

Despite the physiological interconnectedness and the diverse consequences of flight, relationships between flight and the immune system – a physiological system that is essential to survival – remain poorly explored. One study of a long-distance migrant (red knot, *Calidris canutus*) reports no effect of endurance flights on the abilities to simultaneously mount a specific antibody response or to subsequently mount a non-specific response (Hasselquist et al., 2007). More recently, Nebel et al. showed that constitutive immune function decreases slightly following flight by European starlings (*Sturnus vulgaris*) (Nebel et al., 2012). Other studies that explore such interactions with the immune system often do so under the framework of migration, rather than of flight *per se*. For example, Owen and Moore showed that passerines that were experimentally induced into migratory disposition exhibit increased nocturnal activity and decreased responses to a non-specific immune challenge, even though the caged birds were not able to fly freely (Owen and Moore, 2008).

Some biomedical scientists share with ecologists an interest in the effects of activity on immune function, and on this topic, biomedical studies far outnumber ecological ones (reviewed by Walsh et al., 2011; Gleeson, 2007; Petersen and Pedersen, 2005; Pedersen and Hoffman-Goetz, 2000; Nieman and Pedersen, 1999). In essence, this body of work establishes links between short- and long-term exercise and myriad immunological changes. These changes encompass all qualitative possibilities: from acute to chronic, from pro- to anti-inflammatory, and from protective to pathological. Intense short-term exercise by humans leads to rapid but ephemeral changes in leukocyte distributions and activations, increases in cytokine production [particularly muscle-derived interleukin-6 (IL-6)] but not fully developed pro-inflammatory

Table 1. Pigeon races: key characteristics of the events and of the associated birds

| Year | Date | Starting city | Latitude | Longitude | *N* (control/flown) | Hatch-year birds? | Average age (years) | Race distance (km) | Mean time (min) | Mean speed (range) (km h$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 2008 | 21 June | Guxhagen | 51°12′ | 9°29′ | 9/9 | None | ~1.2 | 374 | 369 | 61.2 (52.7–70.1) |
| | 19 July | Aalen | 48°50′ | 10°6′ | 9/9 | None | ~2.1 | 617 | 495 | 76.3 (60.3–91.6) |
| | 24 Aug | Hildesheim | 52°9′ | 9°57′ | 10/6 | All | <1 | 242 | 201 | 72.4 (64.5–75.6) |
| 2009 | 26 July | Würzburg | 49°47′ | 9°56′ | 10/10 | None | ~1.7 | 513 | 418 | 73.8 (67.8–77.9) |
| | 6 Sept | Kassel | 51°19′ | 9°30′ | 10/10 | All | <1 | 339 | 326 | 63.0 (52.0–74.8) |

Hatch-year birds are birds hatched in the same calendar year as their race. These birds are younger and less experienced with racing.

responses, and generalized transient depressions in immune function that do not clearly translate to increased disease susceptibility (Gleeson, 2007; Nieman et al., 2001; Pedersen and Hoffman-Goetz, 2000; Walsh et al., 2011). Exercise can also prompt neuroendocrine changes, including elevations of stress hormones (i.e. glucocorticoids) (Pedersen and Hoffman-Goetz, 2000; Gleeson, 2007).

The flapping-flight of pigeons (*Columba livia*) is a form of intense exercise [see power calculations by Pennycuick (Pennycuick, 1968)]. During races, homing pigeons can fly hundreds of kilometres at speeds of >60 km h$^{-1}$ (Table 1). Researchers have previously used raced homing pigeons to study the costs and consequences of intense flight (Costantini et al., 2008; Bordel and Haase, 1993; Bordel and Haase, 2000). It should be noted that the intense flight performed by homing pigeons is disassociated from the suite of seasonal changes in physiology that typically accompanies the long-distance flights made by free-living migratory birds. This dissociation means that studies of flight by homing pigeons reveal more about the direct consequences of activity and locomotion than about migration. Consequently, less-direct and slower-acting mechanisms of immunological change, such as seasonal hormonal dynamics, can be ruled out, leaving only more-immediate mechanisms to explain any observed differences between flown and un-flown birds. With the racing setup, differences among flown individuals in terms of effort, for example average speed, can be used to further investigate and account for the effects of flight. While our research focused primarily on the physiological mechanisms, our study also bears relevance for a question of interest to avian ecological immunologists studying free-living birds: does sampling of birds after (unknown or unplanned) intense bouts of locomotion affect immunological indices and confound their interpretation? We focused on measuring those indices relevant to and available for use by ecologists working with non-model study species in free-living or captive situations.

We sought to determine what, if any, acute immunological or immune-like responses result from bouts of intense, non-migratory flight. We also compared the effects of flight with the inflammatory effects of a simulated bacterial infection. Specifically, we compared flown and un-flown and endotoxin-injected and un-injected birds in terms of a biomarker of energy use (glucose), an acute phase protein (haptoglobin), non-specific (natural) antibodies, and leukocyte distribution variables. In the subset of birds that were sampled twice, we measured and compared the within-individual changes that occurred in flown and un-flown birds. Finally, in flown birds, we explored the correlations between flight speed and those indices that were affected by flight.

Our hypotheses, based primarily on the biomedical literature (e.g. Pedersen and Hoffman-Goetz, 2000), included the following. (1) If flying under race conditions is intense exercise for homing pigeons, then heterophils should increase post-flight, since patent post-exercise increases occur in the equivalent mammalian leukocyte (i.e. neutrophils). Marked post-flight increases in heterophils and either slight elevations (seen during exercise) or stronger declines (seen post-exercise) in lymphocyte number should lead to elevations in heterophil number relative to the lymphocyte number in flown birds compared with non-flown control birds. (2) Based on post-exercise changes in humans and mammals, eosinophils and monocytes should increase following flight. (3) By analogy with human studies, thrombocytes should increase post-flight because, immediately after exercise, human platelet numbers increase and blood shows ‘hypercoagulability’ (Lippi and Maffulli, 2009). (4) Natural antibody titres should decline post-flight because lymphocyte function and immunoglobulin levels typically fall after intense exercise (see also Nebel et al., 2012). (5) If exercise-induced endotoxemia is the mechanism behind the effects of flight, then post-flight changes should closely mirror post-endotoxin-injection changes, particularly in terms of haptoglobin, which is known to increase in response to endotoxin exposure (van de Crommenacker et al., 2010). Physiological relationships between exercise and sepsis, such as exercise-induced endotoxemia (Brock-Utne et al., 1988; Bosenberg et al., 1988), are known in humans (Petersen and Pedersen, 2005; Pedersen and Hoffman-Goetz, 2000). Discrepancies between the responses to flight and endotoxin challenges would suggest different underlying mechanisms. Any discrepancies might also pinpoint, for ecologists, which of the measured immunological indices are most informative about pathogen-induced inflammation.

## MATERIALS AND METHODS

### Study subjects

We studied the effects of flight in homing pigeons (*Columba livia* Gmelin 1789) of both sexes in 2008 and 2009. In total, we collected samples from 44 flown and 48 un-flown (control) birds. The sampled birds were part of the colony maintained at the Zoological Institute, University of Kiel, Germany (54°20′N, 10°6′E; Animal Experimentation Committee licence nos V312-72241.121-29 and V313-72241.121-29). Living under a ‘restricted free-flight’ regimen, birds took part in daily free-flights of one to two hours and regularly participated in weekend races during the racing season (April to July for adults, July to September for the young). Thus, all flown and un-flown birds in the flight study were considered trained fliers. When not flying, all birds were housed in lofts with exposure to ambient outdoor temperatures and natural photoperiod. Adult males and females were kept in separate lofts, but first-year males and females were kept together. All birds were provided with food (a commercial grain mixture) and water *ad libitum*.

We measured the effects of an endotoxin challenge (lipopolysaccharides from *Salmonella enterica* serotype typhimurium, L7261; Sigma, St Louis, MO, USA) in four male and four female homing pigeons, which were housed at Haren, The Netherlands (53°11′N, 6°36′E). These pigeons were maintained under similar conditions to the birds at Kiel, but they had no access to free-flight. All eight individuals served as their own control in terms of endotoxin effects [University of Groningen, Animal

Experimentation Committee (DEC) licence no. 5095 (van de Crommenacker et al., 2010)].

### Flights and samples

Each flown and control pigeon was associated with one of five races. The five races differed in terms of date, starting point, distance, and other factors (Table 1), but the logistics and handling procedures (e.g. removal from home loft, segregation by sex, etc.) were comparable among races and between flown and control groups (see also Haase et al., 1986; Bordel and Haase, 1993). A local pigeon racing organization arranged the transport and release of all flown birds. Control birds were kept in transport boxes during the same time period. Until the time of release, the flown and control birds had equivalent access to food and water. Upon release of the flown birds, control birds had their sources of food and water removed until blood sample collection was complete.

Immediately (<5 min) upon arrival at the home loft and before they could eat or drink, blood samples were collected from flown birds. The sampling of the control birds was interspersed with the arrival and sampling of the flown birds. We completed blood sampling within 2 min of picking up a bird. We used fresh blood to make blood smears, which were later fixed with methanol and stained (Giemsa stain, modified, GS500; Sigma). Whole blood samples, collected on heparin, were stored on ice until centrifugation, and then the plasma was collected and stored at –20°C until analysis.

In 2008, plasma samples were collected and blood smears were made from birds only at the immediate post-flight (or equivalent control) time point ($t_0$). In 2009, plasma samples were collected at two time points per control/flown bird: immediately post-flight ($t_0$) and again 18 h later ($t_{18}$). Thus, with this subset, we were also able to investigate intra-individual dynamics ($\Delta_{18}$) during this post-flight (or equivalent control) period. In 2009, blood smears were not made. The samples that were used to quantify the effects of endotoxin challenge were also collected at two time points per bird: 30 h pre-challenge (baseline or $t_{pre}$) and 18 h post challenge [endotoxin response or $t_{post}$ (van de Crommenacker et al., 2010)].

### Assays

We quantified glucose concentrations (mmol $l^{-1}$) in 15 µl plasma (flight-study birds) or blood (endotoxin-challenged birds) using a CardioChek PA Analyzer (1708), a small handheld diagnostic device, and PTS Panels Glucose Test Strips (1713; both by Polymer Technology Systems, Indianapolis, IN, USA) (Hegemann et al., 2012a). All measurements fell well within the measuring range of the device (1.11–33.3 mmol $l^{-1}$), as outlined in the manufacturer's instructions.

We measured haptoglobin concentrations (mg $ml^{-1}$) in plasma samples using a commercially available assay (TP801; Tri-Delta Diagnostics, Morris Plains, NJ, USA). Haptoglobin is an acute phase protein that scavenges haemoglobin (Quaye, 2008). Haptoglobin has many functions including limiting the availability of haemoglobin and the iron that it contains from serving as a nutrient for pathogens and an initiator of oxidative damage (Quaye, 2008). When running the assay, which functionally quantifies the haemoglobin-binding capacity of plasma, we followed the manufacturer's instructions with three modifications (Matson et al., 2012). We used twice the amount of plasma per well (15 µl instead of 7.5 µl) and adjusted the calculated concentrations accordingly. A pre-scan at the normal assay wavelength of 630 nm allowed for direct accounting of differences in plasma colour and cloudiness. A second pre-scan at 450 nm enabled us to statistically correct for differences in plasma sample redness, an indicator of hemolysis, which can affect the assay. Each of the three haptoglobin assay plates that were used in this study included an among-plate standard, which was also run in duplicate within each plate [mean within-plate coefficient of variation (CV)=1.6%; mean among-plate CV=1.7%].

We evaluated the haemagglutination and haemolysis titres exhibited by serially diluted plasma samples using the assay of Matson et al. (Matson et al., 2005). Titres reflect $-\log_2$ of the least concentrated dilution step at which rabbit red blood cells (RBA050; HemoStat Laboratories, Dixon, CA, USA) are agglutinated and lysed. Thus, titres gauge two aspects of innate immunity: non-specific natural antibodies (agglutination) and their interaction with complement-like lytic enzymes (lysis). Agglutination was scored from assay plate images recorded 20 min after incubation; lysis was scored from images recorded 90 min and 24 h after incubation (Matson et al., 2012). Blind to sample and plate identity, one researcher (K.D.M.) scored randomized images from each time point at least two times. If the first two scores were <1 titre apart, then we used the mean in analyses. In 3% of cases, the first two scores were ≥1 titre apart; these were scored a third time, and we used the median in analyses. Most samples (99%) from birds in the flight study (both flown and control) showed no lysis; this variable was not analysed further.

From the blood smears, we recorded the relative distributions of six leukocyte types. Blind to sample identity, an independent technician (Cecile Gotteland) evaluated the smears in random order at 1000× magnification with oil immersion. The first 100 leukocytes were counted and classified as heterophils, lymphocytes, monocytes, eosinophils and basophils (Latimer and Bienzle, 2000; Bounous and Stedman, 2000; Campbell, 1995). The numbers of thrombocytes seen while counting these 100 leukocytes were also recorded. No basophils were identified on most smears (89%) from birds in the flight study (both flown and control); this leukocyte type was not analysed further.

### Statistical analyses

All analyses were conducted using R version 2.11.1 (R Development Core Team, 2010). The central goal of these analyses was to establish the effects of intense flight. Flown and control groups were compared in terms of glucose ($t_0$ only), haptoglobin ($t_0$, $t_{18}$, $\Delta_{18}$) and haemagglutination ($t_0$, $t_{18}$, $\Delta_{18}$) using linear models (lm). For purposes of comparison, pre- and post-endotoxin-challenge levels of the same indices were analysed using linear mixed models (lme) with individual identity included as a random factor. Additionally, four leukocyte distribution variables were analysed ($t_0$ only) using generalized linear models (glm, flown *vs* control groups) or linearized mixed models (lmer, pre- *vs* post-endotoxin-challenge) with quasi-binomial error distributions and F-tests. The relationships between heterophils and lymphocytes (henceforth, H|L) were tested (using the cbind function in R) with the minor leukocyte types remaining unaccounted. Monocytes and eosinophils were similarly tested in relation to the numbers of total lymphocytes minus that particular subtype. Thrombocytes, which were counted independently of the other leukocyte subtypes, were tested in relation to total leukocyte number. Three individuals showed extremely high eosinophils (>3 standard deviations from the mean). These individuals were excluded when analysing eosinophil and heterophil distributions since these two cell types can be confused under a light microscope (Jain, 1993). All individuals were included when analysing monocyte and thrombocyte distributions.

In addition to treatment status (i.e. control/flown or baseline/endotoxin-response), we consistently included several biological variables and methodological covariates. Sex (i.e. male or female) was included in all analyses; race (i.e. event identity) was included in all analyses of effects of flight. The index of plasma

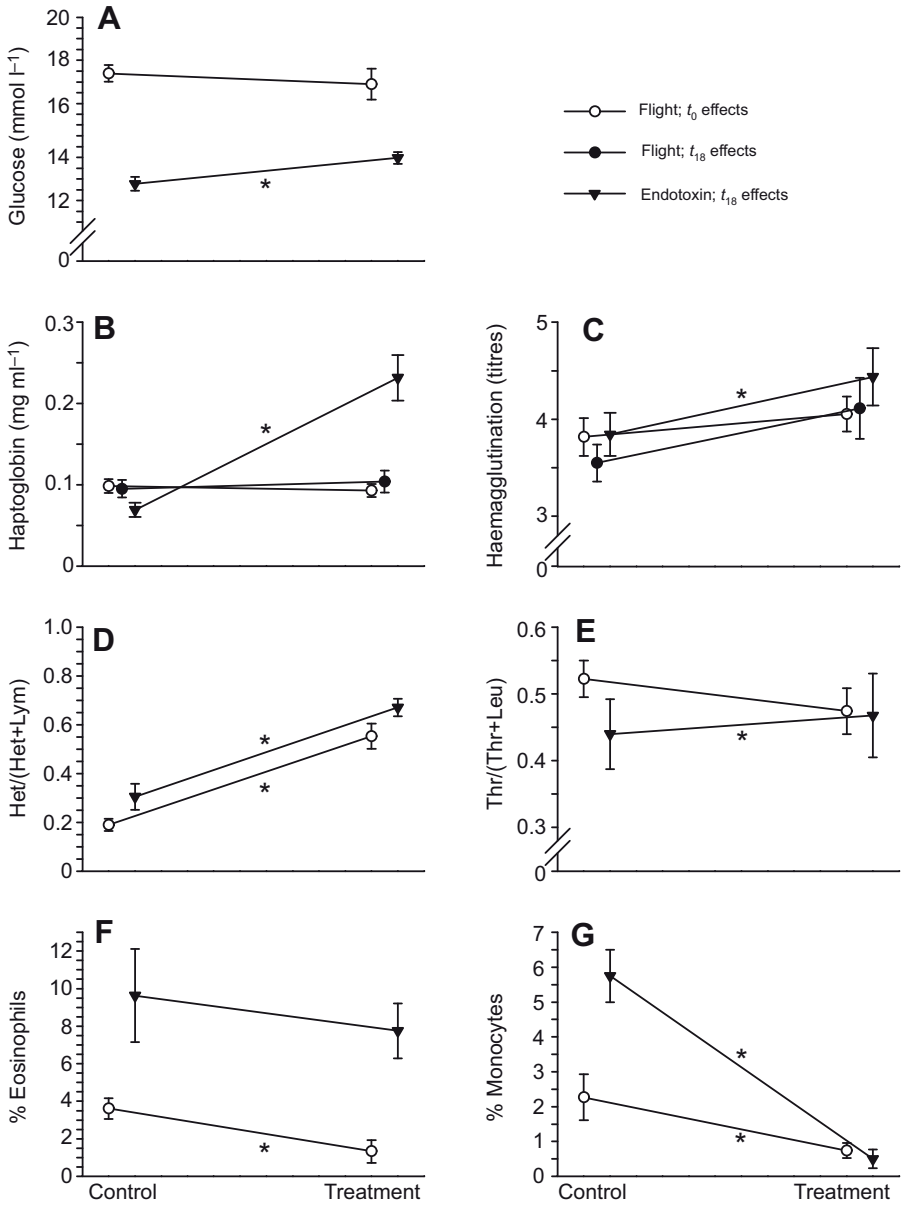


Fig. 1. The effect of flight and endotoxin on (A) glucose concentrations, (B) haptoglobin concentrations, (C) haemagglutination titres, (D) numbers of heterophils relative to numbers of lymphocytes, (E) numbers of thrombocytes relative to numbers of total leukocytes, (F) numbers of eosinophils relative to numbers of remaining leukocytes (i.e. % eosinophils) and (G) numbers of monocytes relative to numbers of remaining leukocytes (i.e. % monocytes). Blood samples were collected at two time points per control/flown bird: immediately post-flight (or equivalent control) time point (Flight; $t_0$ effects) and again 18 h later (Flight; $t_{18}$ effects). Blood samples that were used to quantify the effects of endotoxin challenge were collected 18 h post-challenge (Endotoxin; $t_{18}$ effects). Symbols represent group means, and error bars represent s.e.m. Stars denote a significant effect of treatment (i.e. $P<0.05$). Sample sizes are provided in Tables 2 and 3.

redness (i.e. absorbance at 450 nm) was included in all analyses of haptoglobin. Values at $t_0$ were included in analyses of the effects of flight on $t_{18}$ and $\Delta_{18}$ values. Plasma redness and $t_0$ values were correlated to differing degrees to the other explanatory variables. To abolish this collinearity, standardized residuals of these covariates were calculated from models including the other categorical explanatory variables (i.e. sex, race, treatment); these residuals were employed in place of the original variables.

When tested together and in the presence of all main effects and covariates, the treatment by race and the treatment by sex interactions were never significant at any time point ($t_0$, $t_{18}$, $\Delta_{18}$) in the control/flown dataset (treatment by race in $t_0$ haemagglutination, $F_{4,80}=2.46$, $P=0.052$; all other variables and time points, $0.088<P<0.99$). When similarly tested in the baseline/endotoxin-response dataset, the treatment by sex interaction was significant for three variables: haptoglobin, $\chi^2_1=8.17$, $P=0.004$ (reported in van de Crommenacker et al., 2010); thrombocytes, $\chi^2_1=12.90$, $P<0.001$; eosinophils, $\chi^2_1=4.62$, $P=0.032$. Since the baseline/endotoxin-response study was balanced by sex and since our interest in these interactions was subordinate to our interest in the effects of treatment, all interactions were consistently removed from models when evaluating the overall effects of flight and endotoxin challenge.

When a dependent variable differed significantly between flown and control groups, we explored the effects of flight speed. Only flown birds were included in these analyses, and an index of flight speed replaced treatment (i.e. control/flown). Since flight speeds differed significantly among race events ($F_{4,39}=8.59$, $P<0.001$), we used the difference (in km h$^{-1}$) between an individual's speed and the mean speed of the raced group in which the individual flew. Sex and race were included in all models; standardized values of plasma redness and initial levels of a response variable were included when applicable. The interaction between flight speed and race event identity was also evaluated. This interaction was never significant ($\Delta_{18}$ haptoglobin concentrations, $F_{1,13}=2.92$, $P=0.11$; three $t_0$ leukocyte distribution variables, $F_{2,20}<2.03$, $P>0.15$) and was always removed.

Model assumptions were checked graphically and, in some cases, statistically. In marginal cases, dependent variables were transformed and re-analysed. Transformations improved residual

distributions but never qualitatively altered the results. For the sake of consistency, all presented analyses and results are based on the original data. For all tests, $\alpha$ equalled 0.05.

## RESULTS

### Effects of flight: comparing flown and control birds at 0 and 18 h post-flight

Glucose concentrations, haptoglobin concentrations and haemagglutination titres did not differ significantly between the flown and control birds either at $t_0$ or at $t_{18}$ (all $F \leq 3.7$, $P > 0.06$; Fig. 1; Table 2). At $t_0$, flown birds did exhibit significant elevations in H|L ($F_{1,46}=78.1$, $P<0.001$), significant reductions in eosinophils ($F_{1,46}=9.2$, $P=0.004$), and significant reductions in monocytes ($F_{1,49}=4.3$, $P=0.043$). Thrombocytes did not differ significantly between the flown and control birds at $t_0$ ($F_{1,49}=1.2$, $P=0.27$).

When included in the models testing the effects of flight, several covariates accounted for significant variation. Among all birds in the flight study (i.e. flown and control), males exhibited significantly higher haptoglobin concentrations than females at $t_0$ and $t_{18}$, and race events differed in terms of haptoglobin concentrations and H|L (Table 2). Additionally, plasma redness related positively and significantly to haptoglobin concentrations among all birds in the flight study at $t_0$ ($F_{1,84}=4.1$, $P=0.046$) but not at $t_{18}$ ($F_{1,34}=1.8$, $P=0.20$). Finally, $t_0$ values related positively and significantly to $t_{18}$ values for both haptoglobin ($F_{1,34}=7.2$, $P=0.011$) and haemagglutination ($F_{1,35}=25.1$, $P<0.001$).

### Effects of endotoxin challenge

Glucose concentrations, haptoglobin concentrations and haemagglutination titres significantly increased in response to the endotoxin challenge ($\chi^2_1 \geq 4.7$, $P \leq 0.03$) (Fig. 1, Table 3). After the challenge, birds also exhibited significant elevations in H|L ($\chi^2_1=201.7$, $P<0.001$), significant reductions in monocytes ($\chi^2_1=42.6$, $P<0.001$) and significant elevations in thrombocytes ($\chi^2_1=5.2$, $P=0.022$). Eosinophils did not change in response to the endotoxin challenge ($\chi^2_1=1.8$, $P=0.18$). Males exhibited significantly lower eosinophil numbers than females (Table 3). Plasma redness, included in the model testing the effect of endotoxin on haptoglobin, did not account for significant variation ($\chi^2_1=0.6$, $P=0.43$).

### Intra-individual dynamics: comparing changes in flown and control birds

The intra-individual dynamics of haptoglobin and haemagglutination (Fig. 2) were studied using birds from the 2009 races (Table 1). The change ($\Delta_{18}$) in haptoglobin concentrations between the first ($t_0$) and second ($t_{18}$) bleeds was significantly higher in flown birds than in controls (control, –0.02 mg ml$^{-1}$; flown, 0.01 mg ml$^{-1}$; $F_{1,34}=6.0$, $P=0.020$). Haptoglobin $t_0$ related negatively and significantly to haptoglobin $\Delta_{18}$ ($F_{1,34}=20.3$, $P<0.001$). Races differed significantly ($F_{1,34}=4.8$, $P=0.035$) but sex ($F_{1,34}=0.4$, $P=0.55$) and plasma redness ($F_{1,34}=1.1$, $P=0.30$) had no significant effects.

In contrast, the haemagglutination $\Delta_{18}$ was not significantly affected by flight (control, 0.11 titres; flown, –0.23 titres; $F_{1,35}=1.3$, $P=0.25$). However, as with haptoglobin, there was a significant negative relationship between $t_0$ and $\Delta_{18}$ values of haemagglutination ($F_{1,35}=12.9$, $P=0.001$). Race ($F_{1,35}=0.1$, $P=0.76$) and sex ($F_{1,35}=0.6$, $P=0.44$) had no significant effects.

### Flight speed

Flight speed varied within races among birds; the first bird to return home flew on average 18 km h$^{-1}$ (~25%) faster than the last bird to return (Table 1). The effects of flight speed were studied using all flown birds for which we had a particular flight-sensitive variable (i.e. flown *vs* control). None of these variables showed any evidence of being impacted by relative flight speed: $t_0$ H|L ($F_{1,19}=0.4$, $P=0.54$), $t_0$ eosinophils ($F_{1,19}=0.02$, $P=0.90$), $t_0$ monocytes ($F_{1,22}=0.002$, $P=0.97$), $\Delta_{18}$ haptoglobin concentrations ($F_{1,14}=1.0$, $P=0.34$).

## DISCUSSION

By comparing flown and un-flown homing pigeons, we identified acute effects of intense flight on some immunological indices used by avian ecologists. Some of these effects of flight were comparable to the effects of an injection of endotoxin but other effects were only observed after one of these two physiological challenges. Moreover, the effects of intense flight by pigeons yielded immunologically related changes similar in some regards to intense exercise by humans and other mammals. Regardless of their precise mechanism, these flight-associated changes bear importance for interpretations regarding immunological indices in free-living birds.

### Effects of flight

Flight had significant impacts on H|L (increased), eosinophils (decreased) and monocytes (decreased). Other indices (thrombocytes, glucose concentrations, haptoglobin concentrations and haemagglutination titres) did not differ significantly between flown and control birds. While these results in pigeons partly conflict with our predictions based on the biomedical literature, the elevated H|L exhibited by flown birds was robust and predicted.

The elevated H|L exhibited by flown pigeons is typical of post-exercise changes in leukocyte distributions. In mammals, post-exercise lymphocyte counts drop below pre-exercise counts (Walsh et al., 2011; Pedersen and Hoffman-Goetz, 2000), and the mammalian equivalent of the heterophil (i.e. the neutrophil) increases during and after exercise (Pedersen and Hoffman-Goetz, 2000). These changes in leukocyte distributions have been at least partially attributed to exercise-induced changes in hormone levels, including increases in plasma concentrations of glucocorticoids (Walsh et al., 2011; Hoffman-Goetz and Pedersen, 1994; Pedersen and Hoffman-Goetz, 2000). In birds, heterophils increase and lymphocytes decrease in response to stress and increasing levels of circulating glucocorticoids (Davis et al., 2008; Gross and Siegel, 1983). An array of stressors, including natural variations and experimental manipulations, can affect H|L (Davis et al., 2008). Notably, migrating birds exhibit elevated H|L compared to conspecifics during the breeding season (Owen and Moore, 2006). Changes in H|L that result from exercise and from other stressors may be linked *via* the neuroendocrine system.

Despite the clear effects of flight on H|L, the precise role for a glucocorticoid mediator remains to be determined. In fact, the connections between flight and glucocorticoid concentrations are a bit ambiguous. Studies in pigeons suggest a graduated relationship between flight length and corticosterone concentration (Haase et al., 1986; Viswanathan et al., 1987), which would mirror the relationship between exercise intensity and cortisol concentration in humans (Pedersen and Hoffman-Goetz, 2000). Yet other studies suggest that long flights have no effect on corticosterone concentrations (Hasselquist et al., 2007). Condition-dependent effects are also possible: migrating birds with the biggest energy reserves exhibit the lowest corticosterone concentrations (Gwinner et al., 1992; Jenni et al., 2000). Interestingly, glucose-supplemented human athletes show smaller exercise-induced changes in both cortisol concentrations and leukocyte numbers than non-supplemented athletes (Nieman et al., 2001; Mitchell et al., 1998; Pedersen and Hoffman-Goetz, 2000). More generally, energy balance may be mechanistically important; for example, lymphoid

Table 2. Effects of flight (control/flown), sex (male/female) and race on plasma-based indices and leukocyte distribution variables

| | | | | Flight | | | | Sex | | | | Race | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | *t* | *N* | *n* | Effect: flying | *F* | d.f. | *P* | Effect: being male | *F* | d.f. | *P* | *F* | d.f. | *P* |
| Glucose (mmol $l^{-1}$) | 0 | 3 | 41 (22/19) | –0.50 | 0.4 | 1,36 | 0.549 | 0.33 | 0.1 | 1,36 | 0.702 | 0.229 | 2,36 | 0.797 |
| Haptoglobin (mg $ml^{-1}$) | 0 | 5 | 92 (48/44) | –0.01 | 1.0 | 1,84 | 0.324 | 0.04 | 15.1 | 1,84 | <0.001 | 7.724 | 4,84 | <0.001 |
| | 18 | 2 | 40 (20/20) | 0.01 | 0.5 | 1,34 | 0.471 | 0.03 | 6.4 | 1,34 | 0.017 | 15.824 | 1,34 | <0.001 |
| Haemagglutination (titres) | 0 | 5 | 92 (48/44) | 0.20 | 0.6 | 1,85 | 0.457 | 0.02 | 0.0 | 1,85 | 0.931 | 1.954 | 4,85 | 0.109 |
| | 18 | 2 | 40 (20/20) | 0.56 | 3.7 | 1,35 | 0.062 | 0.11 | 0.1 | 1,35 | 0.709 | 0.015 | 1,35 | 0.904 |
| Heterophil, lymphocyte | 0 | 3 | 51 (27/24) | 1.89 | 78.1 | 1,46 | <0.001 | 0.01 | 0.0 | 1,46 | 0.974 | 23.529 | 2,46 | <0.001 |
| Thrombocyte, leukocyte | 0 | 3 | 54 (27/27) | –0.20 | 1.2 | 1,49 | 0.274 | 0.11 | 0.3 | 1,49 | 0.582 | 1.332 | 2,49 | 0.273 |
| Eosinophil, remainder | 0 | 3 | 51 (27/24) | –1.08 | 9.2 | 1,46 | 0.004 | 0.12 | 0.1 | 1,46 | 0.737 | 0.620 | 2,46 | 0.543 |
| Monocyte, remainder | 0 | 3 | 54 (27/27) | –0.91 | 4.3 | 1,49 | 0.043 | 0.08 | 0.0 | 1,49 | 0.869 | 0.549 | 2,49 | 0.581 |

*N* = number of races; *n* = number of individuals (control/flown).

tissues may be encumbered by a 'glutamine debt' under certain physiologically demanding conditions (Hoffman-Goetz and Pedersen, 1994). Inconsistencies in glucocorticoid responses among species and conditions (e.g. short *vs* long flights, ample *vs* inadequate energy, and migratory *vs* non-migratory disposition) raise questions about what other mechanisms [e.g. changes in other hormones or body temperature (Hoffman-Goetz and Pedersen, 1994)] might promote or limit the effects of flight on the immune system and how these mechanisms are modulated through time.

For some of the measured indices, we obtained only limited insights from previous studies, which led to predictions that were not supported in pigeons. For example, monocytes decreased significantly following flight, contrary to our prediction, which was based upon increases in monocytes following exercise in humans (Nieman et al., 2003; Pedersen and Hoffman-Goetz, 2000; Nieman et al., 2001) and during migration in some passerine birds (Hegemann et al., 2012b). Additionally, thrombocytes were unaffected by flight, even though platelet number and coagulability increase in humans after exercise (Lippi and Maffulli, 2009). These contrasts may reflect deep taxonomic divisions, differences in relative intensity of the exercise, or both. That is, not only are birds intrinsically different from mammals, but the physiological impacts of a race on a homing pigeon may also be quite distinct from the impacts of a marathon on a human, even a well-conditioned one.

Compared to the effects of exercise on lymphocytes, the effects of exercise on plasma proteins are poorly characterized, even in humans (Pedersen and Hoffman-Goetz, 2000). Flown and control birds did not differ significantly in terms of haptoglobin concentration or haemagglutination titres. Limited evidence suggests that transferrin, another (albeit negative) acute phase protein with an iron-binding function, is stable in the short-term (<1 day) following exercise (Pedersen and Hoffman-Goetz, 2000). The stability of haptoglobin concentrations that we observed in pigeons further supports the notion that exercise alone has minimal capacity to induce an acute phase response. A more substantial body of research links intense exercise to declines in dimeric secretory immunoglobulin A [dimeric SIgA (Walsh et al., 2011)]. While natural antibodies [usually pentameric immunoglobulin M (IgM)] and SIgA display some functional parallels (e.g. links to the gut mucosa), natural antibody levels did not decline in flown pigeons. Glucose concentration was also similar between flown and control pigeons; this result confirmed a previous report in pigeons (Bordel and Haase, 1993).

### Comparing physiological challenges: flight *versus* endotoxin

The degree to which responses to intense flight and to endotoxin challenge were analogous differed among variables. Notably, haptoglobin showed extremely divergent responses, and H|L showed virtually identical responses. The similarity observed in the responses of H|L hints at physiological links between flight and endotoxin challenges, conceivably driven by one or more shared mechanisms. For most of the other indices, the relationships between the responses to the two challenges were less distinct, often with both challenges having qualitatively similar effects. Of the possible shared mechanisms (e.g. glucocorticoid and other hormonal responses, inflammatory responses, and endotoxemia) underlying the changes in H|L, hormonal responses seem like a good candidate given the immediateness of the post-flight response. However, as discussed above, the specific flight and physiological conditions that are required to elicit glucocorticoid and other hormonal responses to exercise by birds require further investigation.

The divergent responses of haptoglobin to the two physiological challenges are particularly revelatory and useful in helping to rule out other shared mechanisms. Endotoxin led to large increases in haptoglobin concentrations; flight had no effect. This difference suggests that the suites of changes induced by endotoxin and exercise have distinct mechanistic foundations and pathways. Specifically,

Table 3. Effects of endotoxin injection (control/injected) and sex (male/female) on plasma-based indices and leukocyte distribution variables

| | Endotoxin | | | | Sex | | | |
|---|---|---|---|---|---|---|---|---|
| Variable (*n*=8*) | Effect: injection | $\chi^2$ | d.f. | *P* | Effect: being male | $\chi^2$ | d.f. | *P* |
| Glucose (mmol $l^{-1}$) | 1.20 | 8.6 | 1 | 0.003 | –0.05 | 0.0 | 1 | 0.914 |
| Haptoglobin (mg $ml^{-1}$) | 0.17 | 20.5 | 1 | <0.001 | 0.05 | 3.4 | 1 | 0.066 |
| Haemagglutination (titres) | 0.59 | 4.7 | 1 | 0.030 | –0.22 | 0.3 | 1 | 0.606 |
| Heterophil, lymphocyte | 1.62 | 201.7 | 1 | <0.001 | 0.15 | 0.2 | 1 | 0.660 |
| Thrombocyte, leukocyte | 0.17 | 5.2 | 1 | 0.022 | –0.10 | 0.1 | 1 | 0.741 |
| Eosinophil, remainder | –0.24 | 1.8 | 1 | 0.180 | –0.68 | 4.1 | 1 | 0.042 |
| Monocyte, remainder | –2.50 | 42.6 | 1 | <0.001 | –0.25 | 0.8 | 1 | 0.385 |

*Eight individuals (four male, four female) measured pre- and post-challenge.

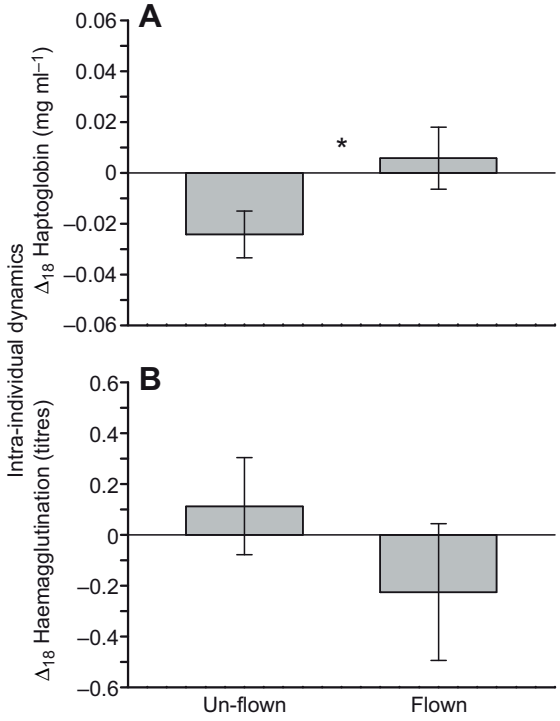


Fig. 2. The intra-individual dynamics, which occurred over the 18 h post-flight (or equivalent control) period (i.e. $\Delta_{18}$), for (A) haptoglobin and (B) haemagglutination. The change in haptoglobin concentrations between the first ($t_0$) and second ($t_{18}$) bleeds was significantly higher in flown birds than in un-flown birds; the change in haemagglutination titres was not different between groups. Bars represent group means, and error bars represent s.e.m. The asterisk denotes a significant effect of flight (i.e. $P<0.05$). The flown and un-flown groups each comprised 20 individuals, which were associated with the two 2009 races.

our study provides no support to the hypothesis that exercise-induced endotoxemia underlies the immunological differences between flown and control birds. In some circumstances, intense exercise has been proposed to result in the leakage of endotoxins across the gastrointestinal epithelia and the induction of systemic responses (Bosenberg et al., 1988; Brock-Utne et al., 1988; Pedersen and Hoffman-Goetz, 2000). However, endotoxin, regardless of origin, should lead to a wide-ranging acute phase response that is characterized in part by increased haptoglobin concentrations. In our study, only direct challenge with endotoxin led to this response (18 h after injection). Haptoglobin levels were not markedly elevated in flown birds relative to un-flown controls either immediately after flight or 18 h later. An alternative explanation is that exercise may not prompt a true systemic inflammatory response, even if muscle cells produce 'inflammation-responsive' IL-6 during exercise (Pedersen and Hoffman-Goetz, 2000). Further investigation of this phenomenon following flight by birds is possible using assays of IL-6 bioactivity (Adelman et al., 2010) or direct measurements of endotoxin in plasma samples.

### Intra-individual dynamics

Our study revealed different intra-individual dynamics for haemagglutination and haptoglobin. In the 18 h that elapsed between collecting the two post-flight samples, on average only small changes (haemagglutination, <0.25 titre or ~5%; haptoglobin, ≤0.02 mg ml$^{-1}$ or ~20%) occurred within individuals. With haemagglutination, these within-individual changes did not differ between flown and control groups. With haptoglobin, the flown group exhibited a significant elevation relative to the control group. With both haemagglutination and haptoglobin in flown and control birds, higher initial values ($t_0$) correlated with smaller changes in the 18 h after a race, suggesting pigeons may face response ceilings that the added challenge of flight does not overcome. Thus, implementing an experimental design that employs dual challenges (e.g. pre- and/or post-flight endotoxin challenges) might provide further insight on the regulation of inflammation.

A range of factors could influence the intra-individual dynamics of haptoglobin. Flown birds seem to have experienced mild inflammations that were either too delayed or too subtle to be detected in the broader flown vs. control comparison. Indirect mechanisms with immunological implications may also be at work. For example, post-flight differences between flown and control groups in terms of energy and water balance might be exaggerated if (as expected) the flown birds consumed more food and water than the control birds after the race. Finally, methodological effects cannot be ruled out: collection of the first blood sample may or may not have affected both groups similarly. Overall, the post-flight intra-individual differences in haptoglobin are very small, and their biological relevance is unclear.

### Flight speed

Our study provided no evidence for relationships between flight speed and the flight-sensitive variables. We focused on speed because other variables of effort, namely flight distance and time, were confounded with each other and with other race event characteristics. Without the use of flight data loggers, no variable of free-flight performance is perfect. For example, later returning (i.e. 'slower') pigeons may have flown detours that could translate to longer distances at equivalent or greater speeds compared with early returning pigeons. Flight distances or times are typically more varied than speeds in wind tunnel studies. In one such study with air speed set at 43.2 km h$^{-1}$, haptoglobin values (standardized by assay plate) correlate negatively with flight times [range=7–431 min (Nebel et al., 2012)].

### Significance for wild immunologists

In the wild, bird flight varies in intensity and timescale. Unless movement is tracked on an individual basis, ecologists can typically only assign broad-brush descriptors about movement ecology (e.g. migrant, resident). But these descriptors are often not very informative about the precise behaviours that precede the collection of blood and other samples in field studies. This study suggests that intense flight immediately prior to blood collection can impact the results of blood-based assays. For example, H|L and other lymphocyte distribution variables were sensitive to flight; other variables, including haptoglobin concentrations, were flight stable. With the strengths and limitations of assaying immunological and physiological function in field studies of wild animals becoming ever clearer (Matson et al., 2012; Horrocks et al., 2011; Millet et al., 2007; Matson et al., 2005; Matson et al., 2006; Buehler et al., 2008; van de Crommenacker et al., 2010), the analysis of flight-sensitive variables still warrants additional precautions. Including covariates of flight or activity level in statistical analyses may be one solution. When controlling for the effects of flight proves to be impossible, investigators may be presented with the challenge of attributing variation in certain leukocyte distribution variables to flight or to some other influential parameter, such as pathogen or parasite exposure. In such cases, measuring one or more flight-stable variables in addition to the flight-sensitive ones may be useful for untangling the effects of flight from the effects of other factors.

Shifting focus from studies of flight *per se* to studies of other types of activity can provide additional insights on the immunological ramifications of physical exertion. Avian ecologists have uncovered connections between extended periods of elevated activity on the one hand and immunological changes and increases in disease susceptibility on the other. This phenomenon is best known from experimental manipulations of parental work load (e.g. Deerenberg et al., 1997; Norris et al., 1994; Ots and Hõrak, 1996). In human athletes, the 'open window' theory links an ephemeral period of immunological change following intense exercise to an elevated risk of post-exercise infections (Walsh et al., 2011; Nieman and Pedersen, 1999). If immunological changes follow intense flight and if these changes open a window of susceptibility in migrating birds, then the physiology of exercise may have important implications for the disease ecology of migration. In any case, understanding how all forms of physical exertion, including flight, influence immunological indices will undoubtedly prove useful in the broader goal of accounting for immunological variation within and among species.

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