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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 (Columbia 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 unflown 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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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

Light and LPS can have similar effects

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

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

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

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
Experimention 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; Borel 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 (t₀). In 2009, plasma samples were collected at two time points per control/flown bird: immediately post-flight (t₀) and again 18 h later (t₁₈). Thus, with this subset, we were also able to investigate intra-individual dynamics (Δ₁₈) 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 t₀₋₃₀) and 18 h post challenge [endotoxin response or t₁₈₋₃₈ (van de Crommenacker et al., 2010)].

**Assays**

We quantified glucose concentrations (mmol l⁻¹) in 15 μl plasma (flight-study birds) or blood (endothoxin-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 –log₂ 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 (t₀ only), haptoglobin (t₀, t₁₈, Δ₁₈) and haemagglutination (t₀, t₁₈, Δ₁₈) 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 (t₀ 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 thromocyte 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
Flight and LPS can have similar effects

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 $\Delta_{t_{18}}$ 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 ($t_0$, $t_{18}$, $\Delta_{t_{18}}$) 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 ($\Delta_{t_{18}}$ 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 \( P \leq 0.7, P > 0.06 \); Fig. 1; Table 2). At \( t_0 \), flown birds did exhibit significant elevations in H/L (\( F_{1,40}=78.1, P=0.001 \)), significant reductions in eosinophils (\( F_{1,40}=9.2, P=0.004 \)), 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 \( t_{18} \, \text{and} \, \text{race events differed in terms of haptoglobin concentrations and H/L} \) (Table 2). Additionally, plasma redness related positively and significantly to haptoglobin concentrations among all birds in the flight study at \( t_0 \) (\( F_{1,44}=4.1, P=0.046 \)) but not at \( t_{18} \) (\( F_{1,49}=1.8, P=0.20 \)). Finally, \( t_0 \) values related positively and significantly to \( t_{18} \) values for both haptoglobin (\( F_{1,43}=7.2, P=0.011 \)) 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 (\( \chi^2=4.7, P=0.03 \)) (Fig. 1, Table 3). After the challenge, birds also exhibited significant elevations in H/L (\( \chi^2=201.7, P<0.001 \)), significant reductions in monocytes (\( \chi^2=42.6, P<0.001 \)) and significant elevations in thrombocytes (\( \chi^2=5.2, P=0.022 \)). Eosinophils did not change in response to the endotoxin challenge (\( \chi^2=1.8, P=0.18 \)). 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 (\( \chi^2=0.6, P=0.43 \)).

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 (\( \Delta t_0 \)) in haptoglobin concentrations between the first (\( t_0 \)) and second (\( t_{18} \)) bleeds was significantly higher in flown birds than in controls (control, \(-0.02 \text{mg ml}^{-1} \); flown, \(0.01 \text{mg ml}^{-1} \); \( F_{1,34}=6.0, P=0.020 \)). Haptoglobin \( t_0 \) related negatively and significantly to haptoglobin \( \Delta t_0 \) (\( F_{1,34}=20.3, P=0.001 \)). 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 \( \Delta t_0 \) was not significantly affected by flight (control, \(0.11 \text{titre} \); flown, \(-0.23 \text{titre} \); \( F_{1,34}=1.3, P=0.25 \)). However, as with haptoglobin, there was a significant negative relationship between \( t_0 \) and \( \Delta t_0 \) 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 (\( F_{1,22}=0.002, P=0.97 \)), \( \Delta t_0 \) 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 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
tissues may be encumbered by a ‘glutamine debt’ under certain physiologically demanding conditions (Hoeman-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 (Hoeman-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 (Niemann et al., 2003; Pedersen and Hoeman-Goetz, 2000; Niemann et al., 2001) and during migratory flight in passerine birds (Hagemann 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 might 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 Hoeman-Goetz, 2000). Flow and control birds did not differ significantly in terms of haptoglobin concentration or hemagglutination titres. Limited evidence suggests that transferrin, an acute phase protein with an iron-binding function, is stable in the short-term (<1 day) following exercise (Pedersen and Hoeman-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 SlgA (Walsh et al., 2011)]. While natural antibodies [usually pentameric immunoglobulin M (IgM)] and SlgA 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 to 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 2. Effects of flight (control/flown), sex (male/female) and race on plasma-based indices and leukocyte distribution variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>t</th>
<th>N</th>
<th>n</th>
<th>Effect: flying F</th>
<th>d.f.</th>
<th>P</th>
<th>Effect: being male F</th>
<th>d.f.</th>
<th>P</th>
<th>Effect: Race F</th>
<th>d.f.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol l⁻¹)</td>
<td>0</td>
<td>3</td>
<td>41</td>
<td>22/19</td>
<td>–0.50</td>
<td>0.4</td>
<td>1.36</td>
<td>0.549</td>
<td>0.033</td>
<td>0.1</td>
<td>1.36</td>
<td>0.702</td>
</tr>
<tr>
<td>Haptoglobin (mg ml⁻¹)</td>
<td>0</td>
<td>5</td>
<td>92</td>
<td>48/44</td>
<td>–0.01</td>
<td>1.0</td>
<td>1.84</td>
<td>0.324</td>
<td>0.04</td>
<td>15.1</td>
<td>1.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2</td>
<td>40</td>
<td>20/20</td>
<td>0.01</td>
<td>0.5</td>
<td>1.34</td>
<td>0.471</td>
<td>0.033</td>
<td>6.4</td>
<td>1.34</td>
<td>0.017</td>
</tr>
<tr>
<td>Haemagglutination (titres)</td>
<td>0</td>
<td>5</td>
<td>92</td>
<td>48/44</td>
<td>0.20</td>
<td>0.6</td>
<td>1.85</td>
<td>0.457</td>
<td>0.02</td>
<td>1.0</td>
<td>1.85</td>
<td>0.931</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2</td>
<td>40</td>
<td>20/20</td>
<td>0.56</td>
<td>3.7</td>
<td>1.35</td>
<td>0.062</td>
<td>0.11</td>
<td>0.1</td>
<td>1.35</td>
<td>0.709</td>
</tr>
<tr>
<td>Heterophil, lymphocyte</td>
<td>0</td>
<td>3</td>
<td>51</td>
<td>27/24</td>
<td>1.89</td>
<td>0.78</td>
<td>1.46</td>
<td>&lt;0.001</td>
<td>0.01</td>
<td>0.1</td>
<td>1.46</td>
<td>0.974</td>
</tr>
<tr>
<td>Thrombocyte, leukocyte</td>
<td>0</td>
<td>3</td>
<td>54</td>
<td>27/27</td>
<td>–0.20</td>
<td>1.2</td>
<td>1.49</td>
<td>0.274</td>
<td>0.11</td>
<td>0.3</td>
<td>1.49</td>
<td>0.582</td>
</tr>
<tr>
<td>Eosinophil, remainder</td>
<td>0</td>
<td>3</td>
<td>51</td>
<td>27/24</td>
<td>–1.08</td>
<td>9.2</td>
<td>1.46</td>
<td>0.004</td>
<td>0.12</td>
<td>0.1</td>
<td>1.46</td>
<td>0.737</td>
</tr>
<tr>
<td>Monocyte, remainder</td>
<td>0</td>
<td>3</td>
<td>54</td>
<td>27/27</td>
<td>–0.91</td>
<td>4.3</td>
<td>1.49</td>
<td>0.043</td>
<td>0.08</td>
<td>0.0</td>
<td>1.49</td>
<td>0.869</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect: injection</th>
<th>Effect: being male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol l⁻¹)</td>
<td>1.20</td>
<td>–0.05</td>
</tr>
<tr>
<td>Haptoglobin (mg ml⁻¹)</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>Haemagglutination (titres)</td>
<td>0.59</td>
<td>–0.22</td>
</tr>
<tr>
<td>Heterophil, lymphocyte</td>
<td>1.62</td>
<td>0.15</td>
</tr>
<tr>
<td>Thrombocyte, leukocyte</td>
<td>0.17</td>
<td>–0.10</td>
</tr>
<tr>
<td>Eosinophil, remainder</td>
<td>–0.24</td>
<td>–0.68</td>
</tr>
<tr>
<td>Monocyte, remainder</td>
<td>–2.50</td>
<td>–0.25</td>
</tr>
</tbody>
</table>

*Eight individuals (four male, four female) measured pre- and post-challenge.
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 (t₀) 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, HIL 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; Horrockes 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 exercise. 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 Hörak, 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 to 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 activity, 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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REFERENCES


