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RESEARCH ARTICLE

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A mimicked bacterial infection prolongs stopover duration in songbirds—but more pronounced in short- than long-distance migrants

Arne Hegemann[1] | Pablo Alcalde Abril[1] | Sissel Sjöberg[1,2] | Rachel Muheim[1] | Thomas Alerstam[1] | Jan-Åke Nilsson[1] | Dennis Hasselquist[1]

[1]Department of Biology, Lund University, Lund, Sweden

[2]Center for Macroecology, Evolution and Climate, Natural History Museum of Denmark, University of Copenhagen, København Ø, Denmark

Correspondence
Arne Hegemann
Email: arne.hegemann@biol.lu.se

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Abstract

1. Migration usually consists of intermittent travel and stopovers, the latter being crucially important for individuals to recover and refuel to successfully complete migration. Quantifying how sickness behaviours influence stopovers is crucial for our understanding of migration ecology and how diseases spread. However, little is known about infections in songbirds, which constitute the majority of avian migrants.
2. We experimentally immune-challenged autumn migrating passerines (both short- and long-distance migrating species) with a simulated bacterial infection. Using an automated radiotelemetry system in the stopover area, we subsequently quantified stopover duration, "bush-level" activity patterns (0.1–30 m) and landscape movements (30–6,000 m).
3. We show that compared to controls, immune-challenged birds prolonged their stopover duration by on average 1.2 days in long-distance and 2.9 days in short-distance migrants, respectively (100%–126% longer than controls, respectively). During the prolonged stopover, the immune-challenged birds kept a high "bush-level" activity (which was unexpected) but reduced their local movements, independent of migration strategy. Baseline immune function, but not blood parasite infections prior to the immune challenge, had a prolonging effect on stopover duration, particularly in long-distance migrants.
4. We conclude that a mimicked bacterial infection does not cause lethargy, per se, but restricts landscape movements and prolongs stopover duration, and that this behavioural response also depends on the status of baseline immune function and migration strategy. This adds a new level to the understanding of how acute inflammation affect migration behaviour and hence the ecology and evolution of migration. Accounting for these effects of bacterial infections will also enable us to fine-tune and apply optimal migration theory. Finally, it will help us predicting how migrating animals may respond to increased pathogen pressure caused by global change.

KEYWORDS
avian, disease ecology, eco-immunology, eco-physiology, stopovers, trade-offs

1 | INTRODUCTION

Billions of birds migrate annually around the globe. While some species make extraordinary long-distance flights, for most species migration consist of intermittent flights (Alerstam, 1990) with most time and energy being spent at stopovers (Hedenström & Alerstam, 1997; Wikelski et al., 2003). Therefore, stopovers determine the overall speed of migration (Lindström & Alerstam, 1992; Nilsson, Klaassen, & Alerstam, 2013) and thus mediate carry-over effects with consequences for the timing of other annual-cycle stages (Schmaljohann, Lisovski, & Bairlein, 2017; Shamoun-Baranes, Bouten, & van Loon, 2010). Hence, stopover duration is crucial for individual fitness, and therefore optimal stopover behaviour is hypothesized to be under strong selection pressure (Hedenström & Alerstam, 1997). While it is known that stopover duration is influenced by weather, fuel deposition rate, predation risk etc. (Lindström, 2003; Newton, 2008; Schmaljohann & Dierschke, 2005), much unexplained variation remains (Jenni & Schaub, 2003; Schmaljohann & Eikenaar, 2017).

Many physiological changes occur in migrants in order to enable a successful migration (Lindström, 2003; Piersma & van Gils, 2011). One such adaptation is modulation of the immune system (Buehler, Tieleman, & Piersma, 2010; Eikenaar & Hegemann, 2016; Owen & Moore, 2008). The immune system protects the body from diseases and is important for survival (Hegemann, Marra, & Tieleman, 2015; Roitt, Brostoff, & Male, 1998). At the same time, it incurs costs in terms of production, maintenance and activation (Hasselquist & Nilsson, 2012; Hegemann, Matson, Versteegh, & Tieleman, 2012; Klasing, 2004). Innate immune responses, and in particular acute phase responses, are costly because they include inflammation and fever as well as in reductions of movements and appetite, usually resulting in body mass loss (Hart, 1988; Hegemann, Matson, Versteegh, et al., 2012; Sköld-Chiriac, Nord, Nilsson, & Hasselquist, 2014). In humans, physical exertion that is comparable to long-distance migration in birds, leads to dampening of immune responses (Gleeson, 2007), and activation of immune responses in songbirds substantially reduces activity during the breeding season (Adelman, Cordoba-Cordoba, Spoelstra, Wikelski, & Hau, 2010; Råberg, Nilsson, Ilmonen, Stjernman, & Hasselquist, 2000). Furthermore, down-regulating immune responses may counteract risks of oxidative stress or autoimmune responses triggered by intense physical activity (Råberg, Grahn, Hasselquist, & Svensson, 1998). Consequently, it has been hypothesized that trade-offs between immune function and refuelling rate exist, and this would increase stopover duration and hence reduce migration speed (Klaassen, Hoye, Nolet, & Buttemer, 2012). Indeed, observational studies suggest that pathogens can impair migration in insects (Bradley & Altizer, 2005), fish (Sjöberg, Petersson, Wickstrom, & Hansson, 2009) and birds (van Gils et al., 2007), and a recent meta-analysis across taxa indicates that parasites and infection intensity negatively impact migration performance (Risely, Klaassen, & Hoye, 2018).

Understanding how individuals move during an infection is arguably a keystone when we want to determine whether and how directly-transmitted diseases spread. Yet, we still know little about how infections modulate stopover ecology. Understanding how individuals move during an infection is critical to determine whether and how directly-transmitted diseases spread, with all the direct implications for livestock and humans (Adelman, Moyers, & Hawley, 2014; Klaassen et al., 2012; Risely et al., 2018). To date, most studies focus on virus-infections (avian influenza in particular) and movements in waterfowl, and many of those find no or negligible effects on migration (Bengtsson et al., 2016; van Dijk, Kleyheeg, et al., 2015; Latorre-Margalef et al., 2009), potentially because low-pathogenic avian influenza does not trigger a strong immune response in waterfowl (van Dijk, Fouchier, Klaassen, & Matson, 2015). Studies on bacterial infections, which trigger an energetically costly acute phase response (Owen-Ashley & Wingfield, 2007) and/or studies on nonwater birds, which represent the vast majority of migrants, are so far missing. Furthermore, understanding the impact of immune challenges on stopover duration, and hence, the speed of migration will also be vital if we aim to predict the possibilities of species to adjust to climate change (Schmaljohann & Both, 2017), as pathogens and diseases are expected to spread northwards with global change (Mills, Gage, & Khan, 2010).

In this study, we experimentally challenged the immune system of six passerine species during autumn migration, using a mimicked bacterial infection to induce an initial (acute phase) innate immune response (i.e., without any proliferating pathogen being present in the birds' bodies). Using automated radiotelemetry, we subsequently compared stopover duration, "bush-level" activity and local movements during the stopover period between control and experimental birds. We also related changes in stopover duration to baseline levels of immune function and to blood parasite infections. We included both long-distance and short-distance migrants in our study to test whether differences between these migration strategies, for example in relation to time- and energy-minimization (Alerstam & Hedenström, 1998), influence the outcome of the experiment. Long-distance migrants have been hypothesized to have a higher susceptibility to diseases, since the physiological demands of long-distance migration may lead to reduced immune function (Klaassen et al., 2012). We hypothesized that birds undergoing an immune challenge will prolong stopover duration and that the effect will be stronger in short-distance migrants as those are under less time constraint than long-distance migrants. We predicted that any increase in stopover duration is independent of previous baseline immune function because an immune response to an infectious agent is so crucial for survival that it overrules baseline immune function (Hegemann, Matson, Versteegh, Villegas, & Tieleman, 2013; Hegemann, Matson, Versteegh, et al., 2012). Finally, we predicted that blood parasite infections should influence the outcome of an immune challenge, because blood parasites can impact the hosts' physiology (Ellis, Kunkel, & Ricklefs, 2014) and thus interact with costs of inducing immune responses.

2 | MATERIALS AND METHODS

We investigated six passerine species during autumn migration in 2014 at Falsterbo Peninsula, a strategic stopover site in Southwest Sweden (55.383°N, 12.816°E). Birds were caught as part of a

standardized ringing scheme in the Falsterbo lighthouse garden where bird density can be extremely high (Karlsson & Bentz, 2004). We studied three long-distance migrants (wintering in sub-Saharan Africa (Cramp, 1988)): Tree Pipit (*Anthus trivialis*; *n* = 20, captured during 29/8–10/9), Willow Warbler (*Phylloscopus trochilus*; *n* = 21, 1/9–21/9) and Common Redstart (*Phoenicurus phoenicurus*; *n* = 21, 2/9–12/9); and three short-distance migrants (wintering in Europe (Cramp, 1988)): Dunnock (*Prunella modularis*; *n* = 20, 12/9–29/9), European Robin (*Erithacus rubecula*; *n* = 20, 26/9–11/10) and Song Thrush (*Turdus philomelos*; *n* = 20, 4/10–14/10). All birds were captured around the peak migration (i.e., close to the median capture date of all individuals during the standardized ringing scheme) of each species, thereby avoiding very early or late migrating individuals (see Supplementary material for capture dates of all birds ringed during the standardized ringing scheme), and in a limited time period to minimize long-term variation in environmental conditions (e.g., food supply, pathogen pressure). All individuals were caught between sunrise and 10.30 and were hatch-year birds except one Tree Pipit and one Redstart. To reduce variation we made every attempt to avoid very lean or very fat birds, thus 108 (89%) out of the 122 individuals had a fat score of 2–4 according to the scale by (Pettersson & Hasselquist, 1985). None of the six species shows a strong socially-driven flocking behaviour during migration. The long-distance migrating species in our dataset are not closely related to each other than to the short-distance migrating species and *vice versa* (see Supplementary material for phylogenetic relationship).

2.1 | Blood sampling

Birds were first measured and ringed by staff from the bird observatory. Afterwards, usually within 30 min after removal from the mist-net and hence before any expected impact of stress on parameters of baseline immune function (Buehler et al., 2008; see also statistic section), we collected blood samples (~100 μl) by puncturing the brachial vein with a sterile needle. Blood samples were kept on ice in an Eppendorf tube until centrifugation for 10 min at 7,000 rpm later the same day. Plasma was separated from RBCs and samples were frozen until subsequent laboratory analysis.

2.2 | Radiotelemetry

To be able to determine stopover duration and quantify behaviour, all birds received coded radio-tags. The five smaller species were tagged with NTQBW-2 Coded Tags (LOTEK, weight 0.3 g). Song thrushes were equipped with MST-720-T transmitters (BIOTRACK, weight 1.4 g). The weight of the transmitters never exceeded 4.2% of the birds' body mass. After cutting some feathers on the birds' back, we glued the transmitters with superglue on the feather stumps (Sjöberg, Alerstam, Akesson, & Muheim, 2017). An automated radiotelemetry system at the Falsterbo peninsula (ca. 6 × 6 km) consisting of three receiver stations (SRX600; Lotek Wireless, Newmarket, ON, Canada) with 4–5 antennas each (in total 13 antennas) and arranged along a line (see Supplementary material for a map) allowed us to estimate stopover duration, departure time and vanishing bearing (hereafter departure direction). We used the stable individual burst rates of the transmitters (2.9–3.1 s) to filter the data by burst rate (Sjöberg et al., 2015). Constant signals over a long period were assumed to be dead birds or transmitters fallen off, and these birds were excluded from the analyses (*n* = 11; eight controls, three experimentals). Stopover duration was calculated as number of days between release time and last recorded signal. By using all signals during the last 10 min from the receiver station that was last in contact with a departing bird, we calculated departure direction as a circular mean (Batschelet, 1981), weighting each signal by signal strength (Sjöberg & Nilsson, 2015). The departure directions were grouped into "forward" (i.e., 135°–315°, SE–NW) or "reverse" (i.e., 315°–135°, NW–SE) migration. For details on the telemetry system set up, transmitters and monitoring regime, see (Sjöberg & Nilsson, 2015; Sjöberg et al., 2015).

The telemetry system also allowed us to calculate two measures of activity. First, we calculated the variance of the signal strength (nonlinear scale, range 30–255) using the function "var" in R and including data from all antennas that detected a given bird in a given time interval (see below). This measure gives an indication of the small-scale activity of a bird (the "bush-level" activity pattern). If a bird sits still (e.g., during the night or during an immune challenge), the variance would be small because the signal strength will be similar throughout. If it moves a lot on the small scale (probably mainly in the range 0.1–30 m), the variance would be high because the signal strength will vary a lot. Second, we calculated the number of antennas that picked up the signal of a bird over a specific sampling period. This measure gives an indication of the local movements of a bird (in the range 30–6,000 m). In this measure, the further a bird moves away from the site of capture and the more it moves around the peninsula, the more antennas will pick up the signal because the receiver stations are arranged along a line. For example, if a tagged bird was registered by more than four antennas it must have left the lighthouse garden, that is the capture site, because there are only four antennas connected to the receiver at the lighthouse. We calculated the activity patterns for three time periods: (a) The first 6 hr after release for each individual bird (and hence endotoxin injection in experimental birds; see below); this period includes only daylight hours for all birds; (b) 14–19 hr after release, that is during the first night for all birds; and (c) 24–30 hr after release (immune challenge in experimental birds), so this period only includes the daylight hours of the next day, but exclude all night-time hours. For any of the periods, we only included birds that stayed at least until the end of the respective period, to exclude activity patterns of birds departing on migration flights.

2.3 | Immune challenge

We alternatingly assigned birds to the control or the experimental group, and attempted to balance control and experimental birds within a day. After blood sampling and transmitter attachment, experimental

birds were injected with 1 μg LPS/1 g body mass dissolved in 2.5 μl PBS subcutaneously. LPS is part of the gram-negative bacteria cell wall and acts as an endotoxin. The injection starts an acute phase response by mimicking the first stages of a bacterial infection without causing an actual (microbe-invasive) infection (Owen-Ashley & Wingfield, 2007). Control birds remained uninjected, because puncturing the skin and injecting a vehicle alone (e.g., PBS only) may also cause inflammation. Consequently, the experimental responses must be viewed as a result of both the LPS and the injection procedure. This combination of effectors does not pose interpretational problems for our study, since our central interest was to induce an innate (inflammatory) immune response and compare this to the absence of such a response, not the effects of LPS per se (see also Hegemann, Matson, Versteegh, et al., 2012; Hegemann et al., 2013; Schultz, Hahn, & Klasing, 2017).

2.4 | Immune assays

To quantify baseline immune function of the birds prior to the immune challenge, we measured three parameters related to innate immune function and one related to the constitutive part of acquired immune function. Innate immune function is an important, generally less specific, first line of defence (Janeway, Travers, Walport, & Shlomchik, 2005), and it is related to natural pathogen pressure (Horrocks et al., 2012, 2015) and responds to environmental conditions and experimental endotoxin injections (Hegemann, Matson, Both, & Tieleman, 2012; Hegemann et al., 2013). Acquired immune function reflects the investment into more specific immune responses that entails antigen-specific protection over longer time-scales (Hasselquist, Wasson, & Winkler, 2001). Specifically, we used three assays to quantify immune function: (a) With a hemolysis-hemagglutination assay, we quantified titres of complement-like lytic enzymes and nonspecific agglutinating (IgM) natural antibodies from preserved plasma samples (Matson, Ricklefs, & Klasing, 2005). Although high baseline lysis is thought to be beneficial in terms of general immune defense, lysis increases following an immune challenge (Hegemann et al., 2013). Agglutination varies between annual-cycle stages (Hegemann, Matson, Both, et al., 2012), but is more genetically controlled than other immune parameters (Versteegh, Helm, Kleynhans, Gwinner, & Tieleman, 2014) and usually unaffected by acute phase (sickness) responses (Hegemann et al., 2013; Matson et al., 2005). Scans of individual samples were randomized among all plates and scored blindly to treatment and migratory strategy. (b) Using a commercially available colorimetric assay kit, we quantified haptoglobin concentrations in plasma samples (Matson, Horrocks, Versteegh, & Tieleman, 2012). Haptoglobin is an acute phase protein that is released from the liver during a pathogenic challenge. (c) With an enzyme-linked immunosorbent assay (ELISA), we quantified the total level of antibodies (mainly nonagglutinating IgY antibodies) (Hegemann, Pardal, & Matson, 2017; Sköld-Chiriac et al., 2014).

2.5 | Molecular analyses of blood parasites

DNA from preserved blood was extracted using standard phenol/chloroform methods (Sambrook, Fritch, & Maniatis, 2002) and subsequently diluted to 25 ng/μl. To determine infection status with the genera *Haemoproteus/Plasmodium* and *Leucocytozoon* a nested PCR amplifying a partial segment of the cytochrome *b* gene was applied using both the Haem-F/Haem-R2 and Haem-FL/Haem-R2L primer pairs (Hellgren, Waldenstrom, & Bensch, 2004). We subsequently grouped the data into birds having no infection, birds having one infection (*Haemoproteus/Plasmodium* or *Leucocytozoon)* or birds having double infections.

2.6 | Statistics

We compared experimental and control groups for each response variable using linear mixed models analysed with the program R version 3.2.3 (R Development Core Team 2015). Even though measurements of baseline immune function are supposed to be unaltered by short-term handling stress (Buehler et al., 2008), we first checked if handling time and immune parameters correlated, but this was not the case for any parameter (always $F < 2.8$, $p > 0.10$). We therefore did not include handling time in further analyses. We then tested if fat had an influence on stopover duration. Probably due to our attempts to reduce variation in fat scores, fat was unrelated to stopover duration (treatment*strategy*fat, $F_{1,98} = 0.001$, $p = 0.95$; treatment*fat, $F_{1,102} = 0.00$; $p = 0.96$; fat, $F_{1,103} = 0.33$, $p = 0.57$). We therefore did not include fat in further analyses. To test whether the immune challenge increased stopover duration, we included the interaction between treatment and migration strategy. We added blood parasite infection status or baseline immune function parameters into a three-way interaction to test whether those parameters influence the variation in stopover duration. To test whether an increase in stopover duration differed in short-distance or in long-distance migrants from the expected length of an acute phase response (i.e., one day Sköld-Chiriac et al., 2014), we calculated for each experimental individual the residual value compared to the stopover duration in control birds and subsequently subtracted one day for the expected acute phase response. In other words, for each experimental individual, we subtracted the average stopover duration of the control group and one day for the expected length of the acute phase response. Then we compared the remaining value against zero. A significant difference in a one-sided *t*-test against zero would indicate that experimental birds prolonged their stopover more than the one day expectation normally induced by an acute phase response. To test for differences in activity patterns (i.e., "bush-level" activity and landscape movements) and for differences in departure direction, we included the two-way interaction between treatment and migration strategy. We used a generalized linear mixed model with Poisson distribution when the number of antennas was the dependent variable, that is when measuring local movements, and with binomial error structure when the departure direction (classified as forward or reverse migration) was the dependent variable. For all models, we included species as random effect (intercept). We always started with the full model and simplified it using backwards elimination based on log-likelihood ratio test with $p < 0.05$ as selection criterion until reaching the minimal adequate model. Graphs with bar-plots were made using the package "GPLOTS" (Warnes, 2009).

3 | RESULTS

3.1 | Behavioural responses to immune challenge

Stopover duration of control birds was on average 1.8 days. Compared to control birds, immune-challenged birds increased their stopover duration by 2 days, and stayed on average 3.8 days ($F_{1,104}$ = 10.2, p = 0.002; see Supporting Information Table S1 for species-specific data and Supporting Information Table S2 for full statistics). In long-distance migrants, the immune-challenged birds stayed 2.4 days thus prolonging their stopover with on average 1.2 days, that is an increase in stopover time of 100% as compared with controls (Figure 1). In short-distance migrants, immune-challenged birds stayed 5.3 days which is a prolongation of their stopover duration with on average 2.9 days, corresponding to a 126% increase in stopover time as compared with controls. After correcting for the stopover duration of control birds and an expected one day delay induced by an acute phase response, in long-distance migrants the increase in stopover duration of immune-challenged birds did not differ from zero (t = 0.42, df = 28, p = 0.34), while in short-distance migrants the increase in stopover duration differed significantly from zero (t = 1.93, df = 28, p = 0.032). This suggests that long-distance migrants on average continued migration directly after the cessation of the acute phase response, while short-distance migrants stayed longer than the expected duration of an acute phase response.

During the first 6 hr, there was no difference in the "bush-level" activity between control and experimental birds ($F_{1,92}$ = 0.35, p = 0.56). However, the immune-challenged birds moved less on the local movement (peninsula) scale than did control birds (F = 4.99, p = 0.025; Figure 2), that is they were less inclined to leave the catching area at the Falsterbo lighthouse to go to new stopover areas on the Falsterbo peninsula. This effect was independent of migration strategy (migration strategy*treatment: F = 0.03, p = 0.87). During the first night and the second day, there was no difference in either "bush-level" activity or local (peninsula) movements between control and experimental birds (all F < 1.8, p > 0.18, Figure 2, Supporting Information Table S2).

The departure direction, categorized as "forward" or "reversed," of immune-challenged birds and control birds did not differ (F = 1.02, p = 0.31).

3.2 | Infection and physiological status

Lysis at the moment of capture, that is prior to the immune challenge, impacted the change in stopover duration after the LPS-injection differently in long- and short-distance migrants, as indicated by a significant three-way interaction ($F_{1,99}$ = 12.8, p < 0.001, Figure 3). In control birds, short-distance migrant birds with high lysis stayed the longest ($F_{1,22}$ = 8.83, β = 1.84, p = 0.007), while in long-distance migrants, we found no relationship between lysis and stopover duration ($F_{1,23}$ = 0.39, β = −0.07, p = 0.54). In immune-challenged birds, the significant relationship between lysis and stopover duration in short-distance migrants disappeared ($F_{1,25}$ = 2.10, β = −0.09, p = 0.16), whereas in long-distance migrants, we found a significant positive relationship between lysis and stopover duration ($F_{1,25}$ = 5.2, β = 0.41, p = 0.031), that is long-distance migrants with higher initial lysis stayed longer after being immune challenged as compared with those having initially low lysis. This means that the LPS-challenge resulted in opposite changes in relationships between short- and long-distance migrants (Figure 3).

Haptoglobin concentrations, agglutination and total IgY levels at the time of capture did not influence the variation in stopover duration and migration strategy (treatment*migration strategy*immune parameter, all F < 2.3, p > 0.13; treatment*immune parameter, all F < 1.5, p > 0.22, Supporting Information Table S2).

Infections with blood parasites did not affect the variation in stopover duration, and this was not affected by migration strategy (treatment*migration strategy*infection, $F_{1,99}$ = 0.06, p = 0.81; treatment*infection, $F_{1,100}$ = 0.01, p = 0.95).

4 | DISCUSSION

We found clear evidence that migrating birds prolong their stopover duration as a response to a mimicked bacterial infection, that is an immune challenge per se. During the first 6 hr after the immune-challenge, that is in a time period that falls fully within the acute phase response, birds reduced their local (peninsula) movements, but apparently did not show lethargy or simply sat still, as the "bush-level" activity did not differ between groups. Furthermore, the physiological state of the immune system (in particular complement activity) before an immune challenge, in interaction with the migratory strategy (long-distance migrants vs. short-distance migrants), had an effect on how much birds increased their stopover duration. Thus, the behavioural response to a (mimicked) infection does not simply overrule the current situation but partly depends both on the baseline status

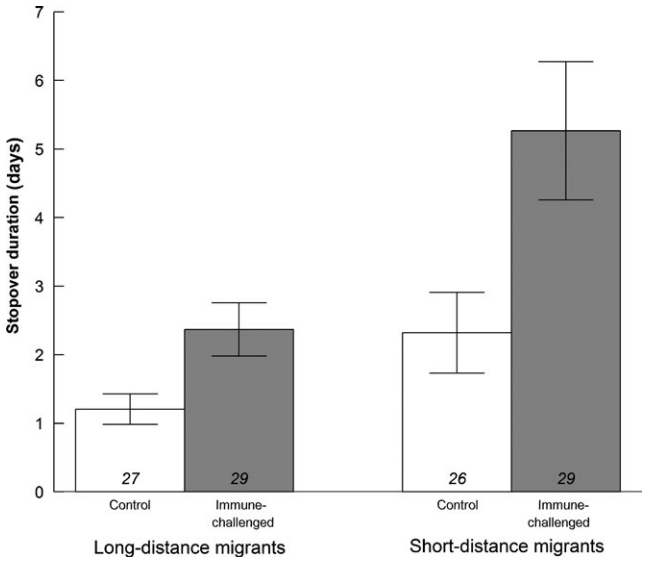

FIGURE 1 Stopover duration (in days) of control and immune-challenged (LPS-injected) individuals of six passerine species at Falsterbo (Sweden). Data are means ± *SEM*; numbers in bars represent sample sizes of individual birds

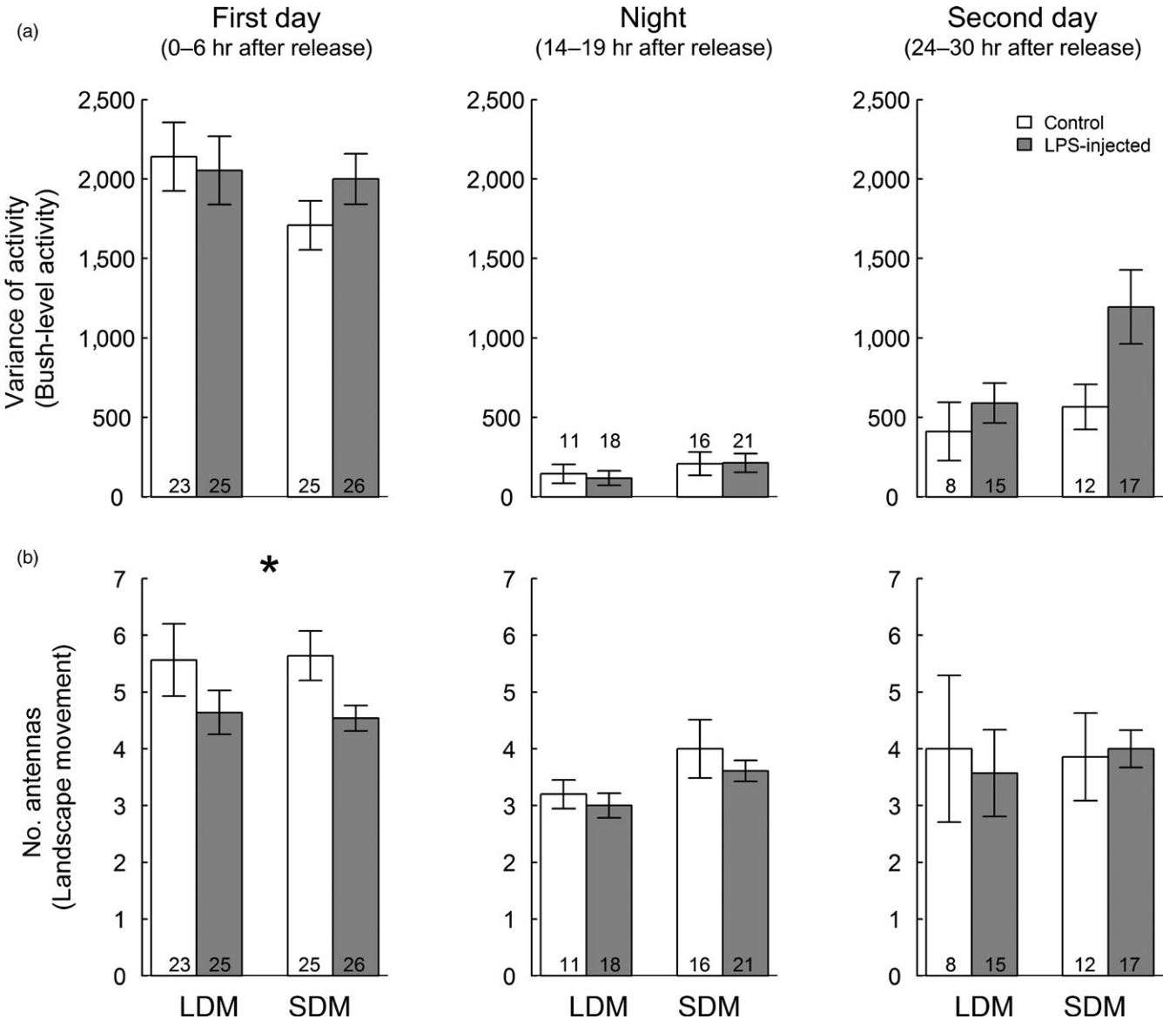

FIGURE 2 Activity patterns of immune challenged and control birds, split into long-distance and short-distance migrants, during autumn migration in Falsterbo. (a) The variance reflects the activity levels on a fine-scale ("bush-level" activity, i.e., 0.1–30 m). Note the very low values in variance of signal strength during the night as compared to during the day, which gives strong support that this measure is useful to indicate activity. (b) The number of antennas that picked up a bird's signal reflects the local movement (i.e., 30–6,000 m) on the stopover site. SDM = short-distance migrants, LDM = long-distance migrants. Data are *M* ± *SEM*; numbers in bars represent sample sizes. The asterisks (*) indicates that groups differ statistically (see results for details)

of the immune system and the migration strategy (which also reflects differences in the remaining migration distance to the wintering quarters).

Contrary to our prediction, the proportional increase in stopover duration (in percentage compared to controls) did only differ slightly and not significantly between long- and short-distance migrants. This supports the hypothesis that an acute phase response is of such importance for survival that it cannot be compromised altogether (Hegemann, Matson, Versteegh, et al., 2012; Hegemann et al., 2013). However, migrants pursuing different migration strategies might still modulate the prolongation of the stopover differently, because immune challenged short-distance migrants more than doubled their stopover duration compared to immune challenged long-distance migrants when measured in absolute time (days). Thus, long-distance migrants may still compromise stopover length (and thus recovery and replenishment of fat deposits) and instead carry on with migration as soon as possible. Our data on increased stopover duration and reduced landscape movements as a consequence of an experimentally-induced innate immune response, contrasts with studies on avian influenza infections in free-ranging mallards (*Anas platyrhynchos*) which found no effect on migratory performance (Latorre-Margalef et al., 2009; Bengtsson et al., 2016; but see van Dijk, Kleyheeg, et al., 2015). This indicates that costs

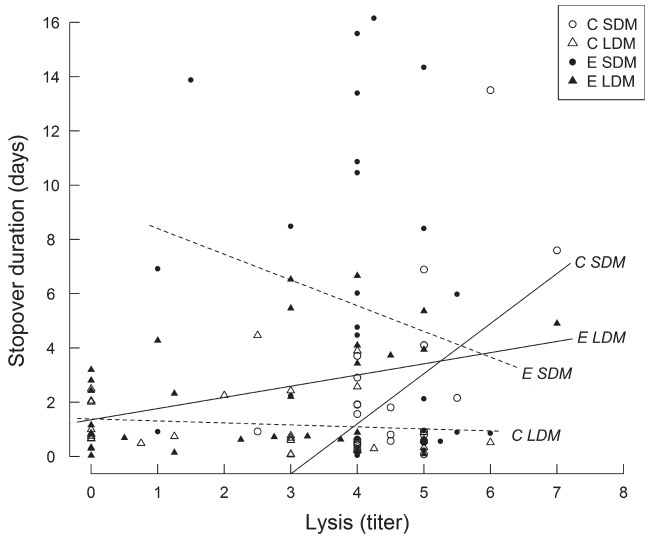

FIGURE 3 Stopover duration (in days) in relation to lysis (titres) at capture (i.e., before immune challenge), treatment and migration strategy in six passerine species at Falsterbo. Solid regression lines indicate significant linear regression between lysis and stopover duration for the respective data subset; dashed regression lines indicate nonsignificant regressions. C = control, E = experimental (LPS-injected), SDM = short-distance migrant, LDM = long-distance migrant

of a mild (low-pathogenic influenza) virus infection are low (van Dijk, Fouchier, et al., 2015) compared to the acute phase response triggered by a (simulated) bacterial infection as in our study. While immune-challenged birds in our study delayed the next migratory flight, Western sandpipers (*Calidris mauri*) flown in a wind tunnel showed no reduction in flight performance after an endotoxin challenge (Nebel, Buehler, MacMillan, & Guglielmo, 2013). However, that study was done on captive birds which experienced no or little constraints and trade-offs and birds paid an immune cost for flying after the flight was completed.

Surprisingly and contrary to our predictions, our "bush-level" activity data suggest that immune-challenged birds did not show detectable signs of lethargy, which is a common behavioural response during an acute phase response to reduce energy expenditure and thereby facilitating a more rapid recovery from acute inflammation (Hart, 1988; Owen-Ashley & Wingfield, 2007). Our data suggest no difference in small-scale movements probably related to feeding activity, which may indicate that time-stressed migrants partly suppress some of the behaviours typically connected to an acute phase response to a bacterial challenge (Adelman et al., 2010). Yet, the immune-challenged birds reduced their local (peninsula) movements during the acute phase response. This reduced local movement was limited to the first day after the challenge, confirming findings from captive birds which showed that fever, another typical aspect of the acute phase response, is limited to the first half-day after the challenge (Marais, Gugushe, Maloney, & Gray, 2011; Sköld-Chiriac, Nord, Tobler, Nilsson, & Hasselquist, 2015). Thus, our immune-challenged birds showed less local movements, meaning that they stayed closer to their capture site. As the capture site is characterized by high bird density, the immune-challenged birds could still suffer from reduced food intake, due to intense competition even if they did not express lethargy and/or anorexia. Because an innate immune response also has direct energetic costs (Hasselquist & Nilsson, 2012; Hegemann, Matson, Versteegh, et al., 2012), the combination of reduced local movements (which potentially reduced their ability to forage optimally) and increased energetic costs suggest that birds may have lost body mass. Yet, the increase in stopover duration was just a little more than one day in long-distance migrants, which is only slightly longer than the supposed length of an acute phase response (Owen-Ashley & Wingfield, 2007; Sköld-Chiriac et al., 2014). Furthermore, immune-challenged birds did not show a higher tendency to conduct reversed migration to reach better refuelling sites than control birds; a behaviour that has been shown for birds in poor body condition (Deutschlander & Muheim, 2009; Lindström & Alerstam, 1986). Taken together, this suggests that there was only limited time for refuelling and restoring lost energy for the long-distance migrants, which is in clear contrast to the almost three-day increase in stopover duration in short-distance migrants. The pronounced increase in stopover duration of short-distance migrants suggests that they prioritized time to recover and refuel after the immune challenge instead of continuing the migration at the next available time, that is the subsequent night for nocturnal migrants and the subsequent morning for diurnal migrants, respectively. Consequently, experimental long-distance migrants may have departed with reduced fuel loads, even though low body condition is known to delay departure (Fusani, Cardinale, Carere, & Goymann, 2009). A continued migration with reduced fuel loads will inevitably have consequences for the subsequent migration. Together with findings that individuals carrying infections can show poorer physical endurance (Bradley & Altizer, 2005), slower migration (Bradley & Altizer, 2005; van Gils et al., 2007) and move shorter distances (Sjöberg et al., 2009), this opens up the possibility that immune responses during migration form a major physiological mechanism for carry-over effects between annual-cycle stages (Studds & Marra, 2005). Effects of infections on migration have also been associated with lower rates of survival (Risely et al., 2018). Hence, this mechanism could also support the idea of migratory culling, that is selective removal of infected hosts during demanding migration journeys (Bradley & Altizer, 2005). Moreover, if birds depart with reduced fuel loads, they will have to make sooner and/or more subsequent stopovers which will also increase the contact with conspecifics and hence the possibility for pathogen transmission if infected with a real pathogen (and not a mimicked infection as in our study).

Interestingly, lysis before the experiment influenced how long birds stayed after the immune challenge, and this effect further depended on the migration strategy. In short-distance migrants, control birds showed longer stopover duration the higher the pre-experimental lysis they had, whereas in long-distance migrants experimental birds showed such a relationship. As lysis

increases during an immune challenge (Hegemann et al., 2013), a positive correlation between stopover duration and lysis in short-distance migrants might suggest that birds with (mild) infections prolong their stopover. The fact that long-distance migrants do not show this pattern supports the hypothesis that long-distance migrants suppress immune reactions to mild diseases during migration (Buehler & Piersma, 2008; Klaassen et al., 2012). Only when additionally confronted with a serious immune challenge (as mimicked by our endotoxin injection), they could apparently no longer suppress an immune response, as it may in a natural situation be crucial for survival (Hegemann et al., 2013). In short-distance migrants, any additional bacterial infection as mimicked by our endotoxin challenge may have resulted in a strong immune response and thus prolonged stopovers independent of previous lysis, hence there was no significant correlation between stopover duration and pre-experimental lysis in immune challenged short-distance migrants.

Blood parasite infections may be such a mild infection that could potentially increase lysis. However, blood parasite infections were not related to lysis in our dataset (Supporting Information Figure S1), and we found no evidence that infections with blood parasites affected the immune challenge. This suggests that blood parasite infections were in their chronic stage and had no major impact on the physiology of the migrating birds. Even though chronic blood parasite infections can have long-term (delayed) fitness consequences (Asghar et al., 2015), effects on short-term physiology are often not visible (Asghar et al., 2015) and are usually only evident during the acute phase (Zehtindjiev et al., 2008). Even for double infections, we found no evidence that those impacted an acute phase response as triggered by our endotoxin injection, despite that others have found severe consequences of mixed infections on other life-history traits in birds (Marzal, Bensch, Reviriego, Balbontin, & de Lope, 2008).

To conclude, we found clear evidence that an infection during migration prolongs stopover duration in both long-distance and short-distance migrants. Our data show that longer stopovers of infected birds are the result of the costs of an immune response per se rather than poor physiological condition of already sick birds. This distinction is important when separating physiological mechanisms behind carry-over effects from effects based on for example low genetic quality or phenotypic (physiological) condition. Additionally, the baseline status of the immune system also influenced the outcome of the immune challenge on stopover duration. Understanding such consequences of acute phase infections will help us to better predict how animals may respond to increased pathogen pressure as well as increasing distances between suitable stopover sites caused by global change (Klaassen et al., 2012). It will also provide us with knowledge of how contracting an infection will affect the migration and stopover strategies of migrating birds and hence help us fine-tuning optimal migration theory (Alerstam, 2011; Alerstam & Lindström, 1990) as time and cost of stopovers are the most important determinants of migration speed and hence migration success. For example, in our study "infected" long-distance migrants prolonged their current stopover period, but only with 1.2 days. Hence, it is possible that the individuals with mimicked infections were stressed and forced to leave too early, which may result in the need for a premature next stopover and maybe an increased number of stopovers during the remaining migratory journey. This may have population-wide consequences, because the likelihood of parasite transmission between bird hosts will increase with number and length of stopovers. Finally, an increase in the time and cost of migration may have potential consequences for fitness (Alerstam & Lindström, 1990). Optimal timing of autumn migration is important as food supply along the migratory route usually deteriorates when season progresses, especially for long-distance migrants (Alerstam & Hedenström, 1998; Newton, 2008). Furthermore, early migrants have competitive advantage in occupying the best territories at stopovers and during winter. This can lead to carry-over effects to the subsequent breeding season (Studds & Marra, 2005). Hence, the timing of autumn migration is under strong selection and delays can have important consequences for individual fitness. Effects may be even stronger during spring migration.

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AUTHORS' CONTRIBUTIONS

A.H., J.Å.N., T.A. and D.H. involved in the study design; A.H. and P.A.A. performed the fieldwork; A.H. and P.A.A. performed the laboratory work; P.A.A., S.S., R.M. and A.H. carried out the analyses of radiotelemetry data; A.H. performed the statistical analyses; all authors involved in the discussion and interpretation of data; A.H. wrote the first draft of the manuscript; all authors gave input on the manuscript and approved the submitted version of the manuscript.

DATA ACCESSIBILITY

Data for this publication are available online at the Dryad Digital Repository: https://doi.org/10.5061/dryad.bb41870 (Hegemann et al., 2018).

ORCID

Arne Hegemann http://orcid.org/0000-0002-3309-9866

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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