Constitutive immune function is not associated with fuel stores in spring migrating passerine birds

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Abstract

Migratory birds may either upregulate their immune system during migration as they might encounter novel pathogens or downregulate their immune system as a consequence of trade-offs with the resource costs of migration. Support for the latter comes not least from a study that reports a positive correlation in autumn migrating birds between fuel stores and parameters of innate and acquired immune function, i.e., energy exhausted migrants appear to have lowered immune function. However, to our knowledge, no study has tested whether this pattern exists in spring migrating birds, which may face other trade-offs than autumn migrants. Here, we investigate if in spring there is a relationship between fuel stores and microbial killing ability, a measure of innate immune function, and total immunoglobulin (IgY), a measure of acquired immune function, in four migrating bird species: Chaffinches (Fringilla coelebs), Dunnocks (Prunella modularis), Song thrushes (Turdus philomelos) and Northern wheatears (Oenanthe oenanthe). We found no correlation between fuel stores and bacterial killing ability or IgY levels in any of the species. However, there was a significant negative correlation between microbial killing ability and Julian date in Song thrushes and Northern wheatears, and between IgY levels and Julian date in Song thrushes. Sex did not affect immune function in any of the species. Our study suggests that the relationship between immune function and fuel stores may be different during spring migration compared to autumn migration. Differences in the speed of migration or pathogen pressure may result in different outcomes of the trade-off between investment in immune function and in migration among the seasons.

Introduction

Every year billions of birds migrate between their wintering and breeding grounds. During these migrations, birds encounter several challenges, which they have evolved to overcome (Maggini et al. 2022). Research shows that birds can regulate physiological processes such as immune function, metabolic rate, and endocrine and neuro-endocrine systems during migration (reviewed by Hegemann et al. 2019). Activation and maintenance of these processes along with long-distance flight is costly and makes migration a highly resource demanding period. In order to keep up with these resource demands, birds are required to stop periodically to deposit fuel (Alerstam and Lindström 1990). A recent review shows that physiological recovery, avoiding adverse weather conditions and spatiotemporal adjustments are also important functions of stopover besides refueling (Schmaljohann et al 2022).

Although stopovers are crucial for successful migration, they may facilitate the spread of diseases and increase the risk of encountering novel pathogens (Todd et al. 2018). Hence, a well-functioning immune system during migration is important to minimize disease-related mortality. However, it has been shown that endurance flight may negatively impact constitutive immune parameters in European Starlings,*Sturnus vulgaris* (Nebel et al. 2012) as well as in Red knots,*Calidris canutus* (Buehler et al. 2010), suggesting lower immunocompetence in birds after a migratory flight. To (partly) compensate for this, migrants are able to boost immune function during stopover (Owen & Moore, 2008), within days of arrival at a stopover site (Eikenaar et al. 2020a, 2023).

If endurance flight during migration negatively affects immune function, while making a stopover positively affects immune function, i.e. helps to recover immune function, then a positive relationship between fuel stores and constitutive immune function of migrating birds at a stopover is expected. Yet, relatively few studies have investigated if there is such a correlation between immune function and migrants' energy condition. A study by Eikenaar et al. (2020b) found that fuel stores are positively correlated with one innate and one acquired parameter of immune function in two sub-species of Wheatears, *Oenanthe oenanthe* during autumn migration. A partly similar result was found during spring migration in four species of thrushes in a study by Owen & Moore (2008) where birds in poor energetic condition had lower leukocyte counts, a measure of acquired immunity, but they did not measure parameters of innate immune function. Hence, more research is needed to determine if this pattern is common among other bird species and if a positive relationships between innate immune function, the important first line of defense, and fuel stores holds true for spring migration, which is much more hurried than autumn migration (Nilsson et al. 2013), mainly due to fewer stopovers in spring (Schmaljohann 2018).

In this study, we investigate if there is a relationship between fuel stores and one parameter of constitutive innate immune function, microbial killing ability, and one parameter of constitutive acquired immune function, the level of immunoglobulins (IgY), in four migrating bird species during spring stopover. These two parameters are relative broad measures of innate and acquired immune function, respectively, and thus reflect an integrative measure of the immune system. We hypothesize that there will be a positive correlation between fuel stores and the immune parameters. We also investigate if the immune parameters differ between species and if there is an effect of time within the migration season on the immune parameters.

Materials and methods

Study site and study species

We studied four migrant bird species, Chaffinches (*Fringilla coelebs*), Dunnocks (*Prunella modularis*), Song thrushes (*Turdus philomelos*) and Northern wheatears (*Oenanthe oenanthe* , Wheatear hereafter), on the Island of Helgoland (54 11'N, 07 °55'E), 50 km off the German North Sea coast. Chaffinches and Dunnocks are diurnal migrants whereas Song thrushes and Wheatears are nocturnal migrants. The birds were caught during daylight hours in the spring (March and April) of 2019. All birds sampled are assumed to be migrants, as only a few (or no) pairs of these species breed on Helgoland (Dierschke et al. 2011).

Capture, sampling, and measurements

All species except wheatears were caught using funnel traps. Wheatears were caught using mealworm baited spring traps. Blood was collected from the wing vein using Na- heparinized microcapillaries. All birds were bled within 10 minutes of capture (range= 1-9.30 minutes, mean= 8.09 minutes), and hence before any expected impacts of handling stress on immune parameters (Zylerberg et al. 2005). The plasma was separated from the blood by centrifugation within 20 minutes of sampling for species caught in funnel traps, and within 2 hours of sampling for the wheatears. The plasma was stored at -20 °C during the field season and at -50 °C later on.

The birds were ringed and measured after blood sampling. Body mass was measured to nearest

0.1g. Fat score was estimated on a scale of 0 (no fat) to 8 (furcular and abdomen bulging, and

breast covered with fat) based on methods described by Kaiser (1993). Muscle score was estimated on a scale of 0 (sharp sternum, muscles depressed) to 3 (sternum difficult to distinguish due to rounded muscles) based on methods described by Bairlein (1994). All the measurements were consistently carried out by a single person. The sex of the birds was determined based on the plumage for Wheatears and Chaffinch, and molecular sexing was performed for Song thrush and Dunnock. All procedures were approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization, Schleswig-Holstein, Germany (permit number V 242-37068/2016).

Laboratory methods

All samples were randomized prior to the lab work and lab work was done blindly with respect to fuel store.

Microbial killing ability

To quantify constitutive innate immune function, the important less specific first line of defence (Janeway, Travers, Walport, & Shlomchik, 2005), we measured microbial killing ability. This assay measures the degree to which an individual's innate immune system can eradicate pathogens, in this case the gram-negative bacteria *Escherichia coli* . It thus represents a broad and integrative measure of innate immune function. We followed the protocol described by French and Neuman-Lee (2012) along with some modifications by Eikenaar and Hegemann (2016). Volumes of 3µl plasma and 4µl of 1.06x10ˆ6 E. coli were finalized as optimal volumes for these species after running several test plates. The absorbance was measured using a FLUOstar Omega microplate reader.

Immunoglobulin (IgY) levels

To measure the constitutive part of acquired immune function we measured immunoglobulin levels. We used the enzyme-linked immunosorbent assay described by Sköld Chiriac et al. (2014) to determine the total level of immunoglobulins (antibodies) in plasma. For the standard curve, a plasma pool from adult jackdaws (*Corvus monedula*) was used. We used a seven-step serial dilution (1000, 250, 62.5, 15.62, 3.90, 0.97, 0.24) for the standard curve. Plasma samples of the focal species was diluted 1:600 and run in triplicate. A BioTeK ELx50 plate washer was used to do the plate washes during the assay and the mean absorbance was quantified using a BioTek EL808 plate reader (measured in 10ˆ3 optical density per minute [mOD/min]). Antibody levels are calculated as the mean of the triplicates of each sample minus the mean value of the blanks and corrected for variation between plates according to the standard curves.

Data analysis

A measure we called "fuel stores" was extracted from the variables fat score and muscle score for each species using principle component analysis. Both fat and muscle scores were included, because migrating passerines depend on both fat (90-95%) and protein (5-10%) for energy during endurance flight (Jenni & Jenni-Eiermann 1998). To test if there is an effect of fuel stores on IgY levels a general linear model was constructed for each species separately. Sex was added as a covariate to the model as immune function can differ between sexes (Arriero et al. 2015). Julian day (date of capture) was added as a covariate as early migrating individuals may differ in immune function from late migrating individuals (Hegemann et al. 2022). Model selection was performed by a stepwise elimination of non-significant variables by using the drop.1 function in R. To test if there were differences in the level of immunoglobulins between the four species, a one-way ANOVA along with the Tukey test was performed. The microbial killing ability data was not normally distributed. Therefore, Spearman's correlations were performed to test whether microbial killing ability is correlated with fuel stores and Julian day. A Kruskal-Wallis test along with a pairwise Wilcoxon test was performed to test for sex and species differences in microbial killing ability.

Results

There was no significant correlation between fuel stores and microbial killing ability (Table 1, Figure 1) or IgY levels (Table 2, Figure 1) in any of the four species. However, Julian day (date of capture) had a significant negative effect on microbial killing ability in Song thrushes and Wheatears (Figure 2, Table 1) and on IgY levels in Song thrushes (Figure 2, Table 2), meaning birds arriving at the stopover later in the migration season had lower microbial killing and lower IgY levels ability than birds arriving earlier. Microbial killing ability and IgY levels did not differ between sexes.

Table 1: The correlation test output between microbial killing ability and fuel stores, microbial killing ability and Julian day in four species of migrating birds during spring migration; and Wilcoxon test output for sex

differences in microbial killing ability in four species during spring migration. P-values <0.05 are marked in bold.

Species	Test	Statistical pa
Chaffinch Fuel stores Julian day Sex	Spearman's correlation Spearman's correlation Wilcoxon's test	rho= 0.107 rh
Dunnock Fuel stores Julian day Sex	Spearman's correlation Spearman's correlation Wilcoxon's test	rho= 0.092 rh
Song thrush Fuel stores **Julian day** Sex	Spearman's correlation Spearman's correlation Wilcoxon's test	rho= 0.110 rh
Wheatear Fuel stores **Julian day** Sex	Spearman's correlation Spearman's correlation Wilcoxon's test	rho= 0.003 rh

Table 2: General linear model output for models testing the effects of fuel stores, sex, and Julian day on IgY levels in four bird species during spring migration. Significant effects are marked in bold. Statistical parameters displayed are from steps prior to dropping effects from a model.

Model/Species	Estimate	Standard Error	T-value	P-value
Chaffinch Fuel stores Sex Julian day	0.448 1.761 0.490	0.859 2.540 0.287	0.521 0.693 1.707	0.605 0.491 0.0
Dunnock Fuel stores Sex Julian day	1.588 1.920 0.017	0.909 1.986 0.181	1.784 0.967 0.099	0.087 0.339 0.9
Song thrush Fuel stores Sex **Julian day**	-1.323 -2.526 -0.288	0.949 2.017 0.139	-1.395 -1.252 -2.071	0.170 0.217 **0.0**
Wheatear Fuel stores *Sex* Julian day	0.900 -2.724 0.193	0.705 1.604 0.174	1.277 -1.604 1.109	0.210 0.118 0.2

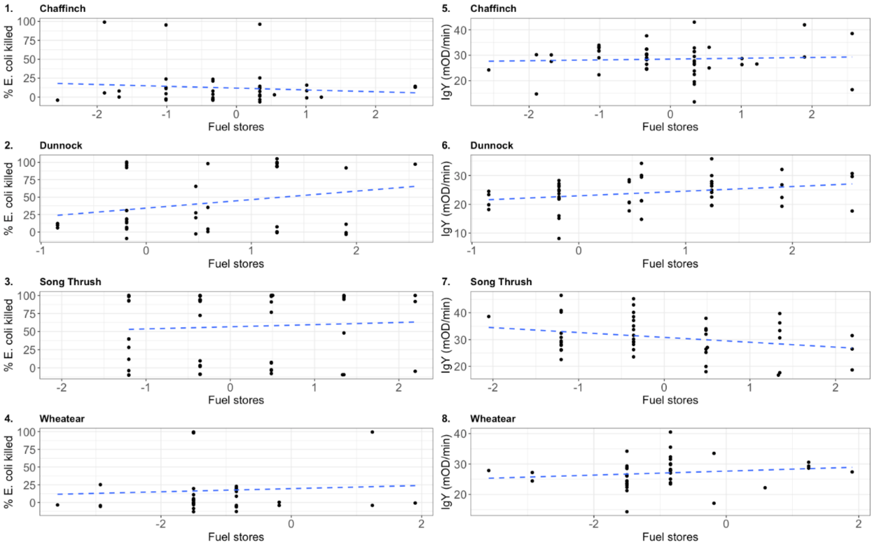

Figure 1: Scatter plots of immune function and fuel stores in four bird species during spring migration. Panels 1-4 show the relationship between microbial killing ability (% E.coli killed) and fuel stores and panels 5-8 show the relationship between IgY levels (mOD/min) and fuel stores. Dashed lines indicate non-significant correlations. Please note that the x-axis is differently scaled among the figure panels.

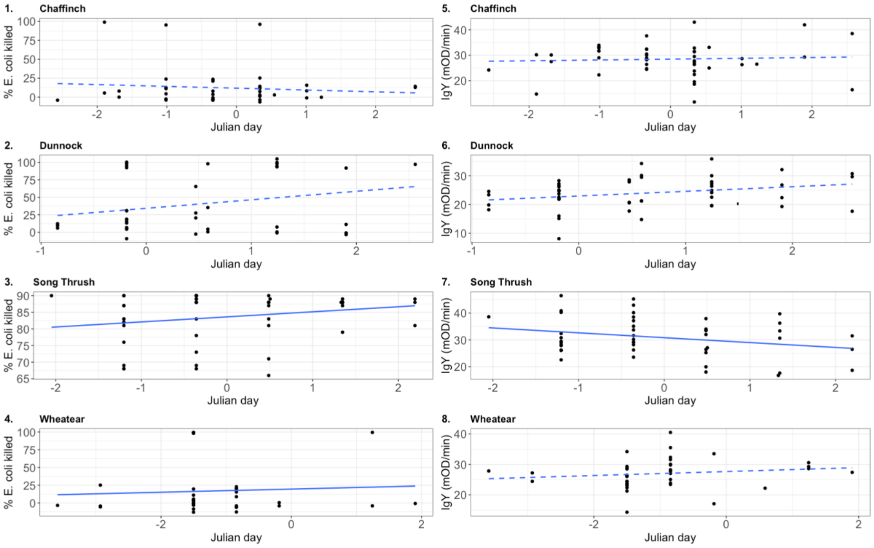

Figure 2: Scatter plots of immune function and Julian day in four bird species during spring migration. Panels 1-4 show the relationship between microbial killing ability (% E.coli killed) and Julian day and panels 5-8 show the relationship between IgY levels (mOD/min) and Julian day. Dashed lines indicate non-significant correlations. Significant relationships are depicted in bold lines in the panels 3, 4 and 7 respectively. Please note that the x-axis is differently scaled among the figure panels

Microbial killing ability was significantly different between the four species (Figure 3, chi-squared= 20.43, df= 3, P<0.001). A pairwise Wilcoxon test showed significantly higher microbial killing ability in Dunnocks when compared to Chaffinches (P=0.004) and Wheatears (P=0.009). Song thrushes showed a significantly higher microbial killing ability than Chaffinches (P=0.004) and Wheatears (P=0.009). There was no significant difference in microbial killing ability between Wheatears and Chaffinches (P=0.59), and Dunnocks and Song thrushes (P=0.59). Similarly, immunoglobulin levels were significantly different between the four species (f=11.25, df= 3, P< 0.001, Figure 3). A Tukey test revealed that the IgY levels in Dunnocks were significantly lower than IgY levels in Chaffinches (P=0.001) and Song Thrushes (P<=0.001). The IgY levels in Wheatears were significantly lower than that in Song thrushes (P=0.01). There was no difference in levels of IgY between Song thrushes and Chaffinches (P=0.19), Wheatears and Chaffinches (P=0.68) or Wheatears and Dunnocks (P=0.07).

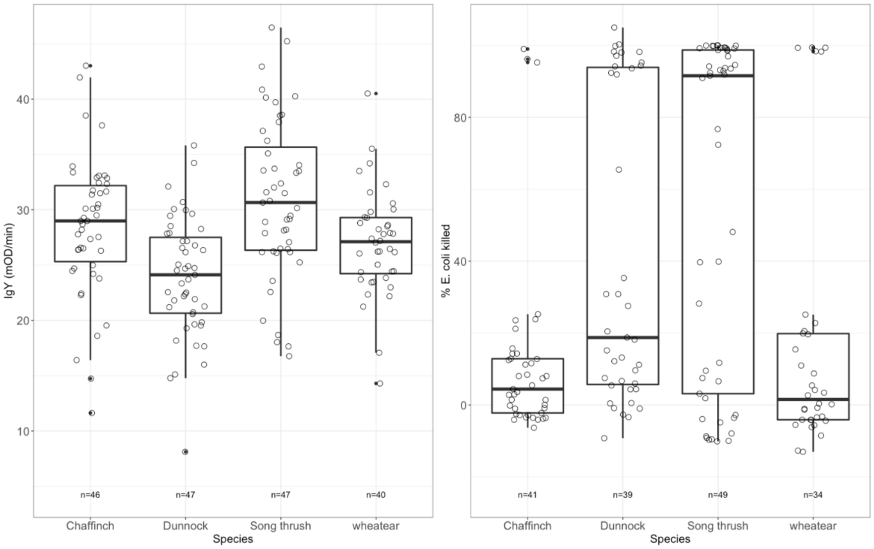

Figure 3: Box plots showing differences in IgY levels (mOD/min) and bacterial killing ability (% E.coli killed) between the species. The dots in bar plot show the distribution of measurements and lines indicate the lower quartile, median and upper quartile within each species.

Discussion

We found that fuel stores were not correlated with an integrative measure of constitutive innate immune function (microbial killing ability) nor with a measure of constitutive acquired immune function (IgY levels) in any of the four species during spring migration. This is contrary to what was found in wheatears during autumn migration where there was a positive correlation between fuel stores and both these two same parameters of immune function (Eikenaar et al 2020b). Interestingly and similar to this current study, there was no correlation between energetic condition and immunoglobulin levels in spring migrating thrushes in the Americas either (Owen & Moore 2008). However, Owen & Moore (2008) did observed a negative relationship between energetic condition and total White blood cell count as well as one out of five leukocyte types. They did not measure innate immune function. Taking together those studies may suggest that while there is a correlation between immune function and fuel stores during autumn migration, such a relationship may at most be weak in spring migrating birds. A potential explanation for the differences between autumn and spring migrating birds could lie in seasonal differences in migration speed that impact resource allocation between immune function and the demands of migration. There is evidence that progress during spring migration is much faster than during autumn migration as birds travel with fewer stopovers in spring (Nilsson et al. 2013; Schmaljohann 2018). The reason for a more hurried spring migration is that there is a fitness advantage for arriving early at the breeding grounds; individuals that arrive earlier at the breeding grounds are more successful in finding a territory and a mate (Kokko 1999, Aebischer et al. 1996). Perhaps, spring migrants tend to invest their resources (energy and nutrients for fuel) more into timely arrival at the breeding site rather than in immune function compared to autumn migrants. Fewer stopovers may also mean that spring migrants have less opportunity to recover their immune function during stopover after strenuous endurance flights (Eikenaar et al. 2020a; 2023). Some support for a seasonal difference in immune function comes from comparing values from the current and previous studies. Eikenaar et al (2020b) found that in autumn migrating Wheatears IgY levels ranged from 45-60 mOD/min in autumn, whereas in the current spring study these typically ranged from 20-35 mOD/min (Figure 3). Similarly, in comparison to an autumnal study by Hegemann et al. (2018), the mean IgY levels for Dunnocks, Song thrushes and Chaffinches were much lower in the current spring study. It is important to note though that direct comparisons should be made with caution, as the assays in the various studies were performed with different standards and dilutions. Nonetheless, they are suggestive of a lower (investment in) immune function in spring than autumn. which could help to understand why in the current spring stopover study we did not observe relationships between migrants' fuel stores and parameters of immune function.

Three other, not mutually exclusive hypotheses might further help explain why there is no correlation between immune function and fuel loads in spring migrating birds and why they may have lower immune function than birds during autumn migration. All three relate to pathogen pressure, i.e. the risk of encountering pathogens and becoming sick. First, densities of birds is usually much lower in spring than during autumn, especially when considering that our study location is relatively close to the breeding locations, because most annual mortality happens between autumn and spring (Sillett & Holms 2002, Leyer et al. 2013, Klaaassen et al. 2013). Lower densities of other birds will decrease contact rate and hence the risk of transmission of infectious diseases. Second, prevalence of diseases is often higher during autumn than during spring (Latorre-Margalef et al. 2014, van Dijk et al. 2014), which also reduces the risk of infection. Third, during autumn birds are on their way to southerly wintering grounds, which supposedly harbor more pathogens than the northerly breeding grounds to which birds are heading to during spring (Westerdahl et al., 2014; O'Connor et al., 2020). All three hypotheses reduce the risk of disease contact and hence the need for strong constitutive immune function (Horrocks et al. 2011, 2015, Hegemann et al. 2012). Hence this may allow birds to invest more in migration and less in immune function during spring migration compared to autumn migration.

We did observe a relationship between immune function and Julian day in some species; individuals that arrived on Helgoland early in the migration season had higher bacterial killing ability in case of Song thrushes and Wheatears and higher levels of IgY in case of Song thrushes. We can only speculate on the reason(s) for these relationships, but perhaps birds arriving at stopover earlier are of superior quality than the ones arriving later. It is also possible that individuals wintering further South passed by Helgoland later, and had lower immune function because of their longer migratory flights. We furthermore observed significant differences in microbial killing ability as well as IgY levels among the species, which is in line with previous studies (e.g. Tieleman et al 2015; Hegemann et al. 2022; Eikenaar et al. 2023). Variation in immune function between species can have multiple reasons including differences in risk of infection, phylogenetic history, migration strategy etc. It is beyond the scope of our study to discuss or even disentangle possible reasons, but the data provided might prove valuable for future studies or meta analyses.

In conclusion, our study showed that unlike during autumn migration, during spring migration fuel stores are not related to immune function. Moreover, the current and previous studies hint at differential seasonal investment in immune function, which could be further investigated in focused comparative studies.

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Data accessibility statement

The data that support the findings of this study are openly available in dryad at https://doi.org/10.5061/dryad.h44j0zpqv

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