ECOPHYSIOLOGICAL ADAPTATIONS ASSOCIATED WITH ANIMAL MIGRATION

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Table of Contents

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Editorial: Ecophysiological adaptations associated with animal migration

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Editorial on the Research Topic
Ecophysiological adaptations associated with animal migration

Seasonal migration to track favorable environmental conditions and resources is a widespread strategy across all animal taxa, including insects, fish, birds, and mammals. Migration distances can range from meters to tens of thousands of kilometers (Hansson and Åkesson, 2014). This period is often resource-demanding (Sapir et al., 2011) and migrants regularly encounter challenging conditions en route, including extreme and/or fluctuating temperature, humidity, solar radiation, osmotic stress, pathogen pressure, pollution, and hypoxia (Figure 1). Migrants cope with these challenges *via* a multitude of physiological adaptations (e.g., McCormick and Saunders, 1987; Piersma and Van Gils, 2011; Gwinner, 2012; Cooper-Mullin and McWilliams, 2016; Hegemann et al., 2019), which can conflict with other important annual-cycle and life-history events such as growth, reproduction and molt. Migration therefore represents one of the most significant physiological challenges in the life of an animal, yet our understanding of the interplay between different physiological adaptations and the currencies involved (energy, nutrients, time, etc.) is still limited (Hegemann et al., 2019). To better understand the ecology and evolution of migration and to highlight important directions for future work, this Research Topic presents studies demonstrating recent advancements in our knowledge of the ecophysiological underpinnings of migration at different stages and scales.

Preparing for migration

Animals typically undergo dramatic physiological and behavioral changes in preparation for migration (McCormick and Saunders, 1987; Piersma and Van Gils, 2011), with the accumulation of energy reserves being particularly important for endurance migrants (Guglielmo, 2018). Many species accumulate significant amounts of fuel before their seasonal migration starts and this is usually controlled by endogenous programs (Maggini and Bairlein, 2010; Maggini et al., 2017). Cornelius et al. show that even facultative migrants, i.e., animals that migrate irregularly in response to changing conditions, can quickly increase their fat stores when necessary.

Other changes prior to migration often include reorganization of body composition to reduce maintenance costs for tissues not necessary during endurance exercise (Piersma et al., 1999; Piersma and Van Gils, 2011). Piersma et al. show that Bar-tailed Godwits (*Limosa lapponica*) shrink their digestive tract but maintain a higher relative size of their heart and flight muscles while accumulating fuel stores. This poses a particular challenge to animals that stop to replenish depleted fuel stores during migration. Refueling at stopover sites starts at a low rate (i.e., little food is converted to body mass) to accumulate lean mass, followed by a hyperphagic phase to accumulate fat stores, and a final phase to reallocate protein from the digestive system to flight muscle synthesis to facilitate further migration (Handby et al.; Guglielmo et al.). These processes have been studied most intensively in birds, and further work is needed to confirm that similar dramatic changes in body composition occur in other migratory animals.

Dealing with variable or changing abiotic conditions along the route

Migration poses countless challenges and mortality is highest during the migratory period in many animals (e.g., Sillett and Holmes, 2002; Klaassen et al., 2014; Kantola et al., 2019). Individuals must be prepared to meet high energetic demands and cope with additional environmental challenges, which can vary substantially along migratory routes (Figure 1). One of the most predictable abiotic changes migrants can experience as they move across geographic areas is a change in photoperiod, somewhat akin to 'jet lag' in humans, and requiring re-organization of circadian rhythms. Two studies in this Research Topic show that migratory animals are particularly proficient in responding to changing photoperiod, both in the circadian rhythms of body temperature in migrating geese (Eichhorn et al.) and daily migratory activity in two passerine species (Åkesson et al.).

Migrating animals often cross ecological barriers, where little to no opportunities for resting or refueling may be available. Successful crossing of these barriers can be highly dependent on current environmental conditions, thus requiring individuals to carefully choose when to embark on the journey (Fransson et al., 2008; Deppe et al., 2015; Dossman et al., 2016). An impressive example for long barrier crossings among insects is the globe skimmer dragonfly (*Pantala flavescens*), which is hypothesized to migrate from India to East Africa by crossing the Indian Ocean. Hedlund et al. used an energetic flight model and wind trajectory analysis to show that this transoceanic migration is possible, but likely requires the capacity to select favorable winds.

Environmental conditions can also be unpredictable, including extreme weather events or challenging temperatures. Snow buntings (*Plectrophenax nivalis*), for example, maintain cold endurance during spring migration, presumably to cope with cold temperatures en route and at their Arctic breeding grounds (Le Pogam et al.). On the other end of the spectrum, desert-crossing passerines facing high temperatures during stopover adjust their activity patterns according to their species-specific dehydration risk (Paces et al.). These results show that flexible behavioral and physiological responses to challenging abiotic conditions are crucial for migrating animals to successfully complete migration.

Physiological trade-offs to facilitate successful migration

Coping with the high resource costs of migration often requires physiological trade-offs with other traits, such as growth, breeding and predator evasion. To deal with these trade-offs, two main strategies can be used: (i) minimizing the time spent migrating, i.e., traveling fast even if expensive, and (ii) minimize the total energy cost of migration, i.e., traveling at speeds that conserve energy (Alerstam and Lindström, 1990; Hedenström and Alerstam, 1997; Alerstam et al., 2003). Clerc et al. use two migrating bat species in which both time-minimizers and energy-minimizers co-occur within the same population, providing an excellent study system to test predictions. Using several cutting-edge techniques to estimate energetic state, they demonstrate that the two strategies can extend to the intraspecific level, as migratory strategies seem to vary between sexes, and that thermoregulation (specifically torpor in bats) might be an important regulator of stopover decisions.

During migration, animals often have to reallocate resources and this may affect several physiological systems including the immune system (Buehler et al., 2010). The immune system is important for survival (e.g., Wilcoxen et al., 2010; Hegemann et al., 2015; Roast et al., 2020), particularly in migrants which cope with novel diseases and high risk of disease transmission (Altizer et al., 2011; Westerdahl et al., 2014). Yet, trade-offs with other demands of migration can lead to modulation of immune function while migrating (e.g., Owen and Moore, 2006,

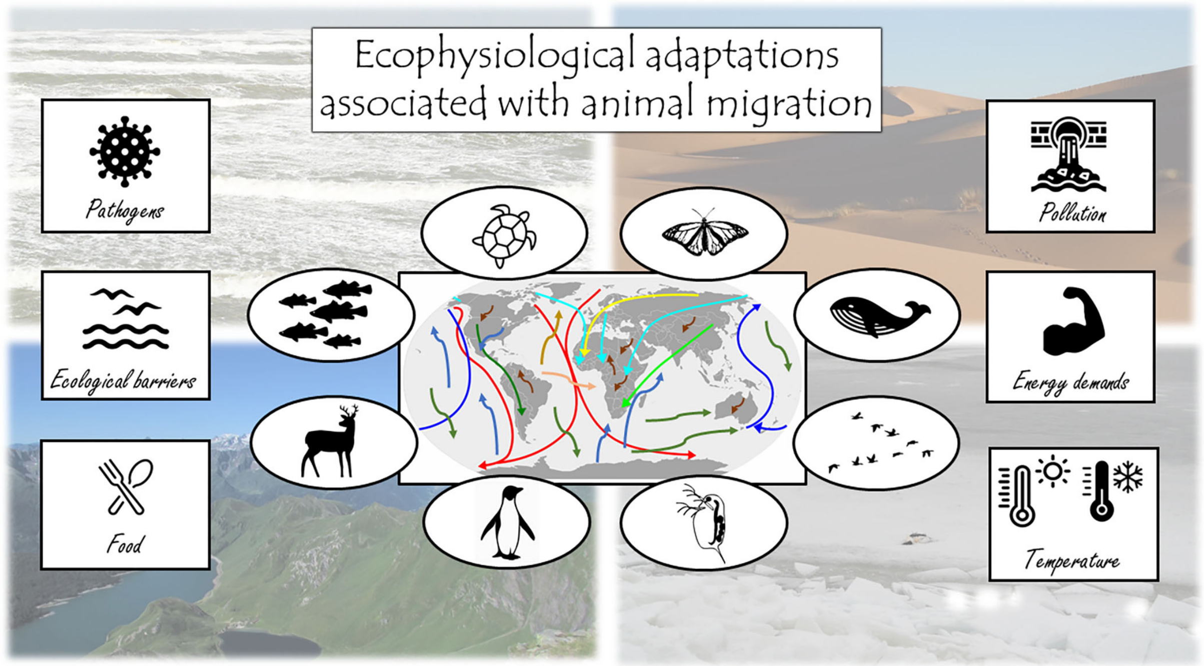

FIGURE 1
Migrating animals of all taxa experience a multitude of challenges during their seasonal migrations. In this Research Topic, we collected 14 papers that investigate several of those challenges in different taxa including insects, fish, birds and mammals.

2008; Buehler et al., 2008, 2010; Eikenaar and Hegemann, 2016; Hegemann et al., 2018; Eikenaar et al., 2020). Hegemann et al. extend this knowledge by showing that immune function differs among species and among individuals depending on migratory strategy and timing. Nevertheless, migrants are exposed to varying pathogen landscapes as they move (Altizer et al., 2011; Westerdahl et al., 2014; Hall et al., 2016), which may compound the physiological challenges already experienced. For example, gut parasites have been thought to disrupt osmoregulation in salmon moving from fresh- to saltwater, but Finlay et al. did not find such effects, suggesting that fish either cope with the presence of parasites or that negative effects become apparent at a later stage of infection. Further studies investigating varying immune function and its interactions with infection rates are of paramount importance to understand how parasite and disease exposure influence migratory animals (Binning et al., 2022), particularly in the context of predicting future disease outbreaks and pandemics (Carlson et al., 2022).

Endurance exercise such as migration can also lead to relatively high oxidative stress (Costantini et al., 2008, 2019; Jenni-Eiermann et al., 2014), necessitating appropriate defense mechanisms (Eikenaar et al., 2022). McWilliams et al. provide a comprehensive review showing that birds can adjust their diet to augment their defenses by, for example, consuming foods rich in antioxidants. Furthermore, Cooper-Mullin and McWilliams show in a field experiment that recovering from oxidative damage may be an important function of migratory stopovers in addition to refueling. These studies further illustrate the complexity of the physiological adaptations required for animals to successfully complete migrations.

Conclusions and outlook

Migration allows many species to survive and thrive in dynamic environmental conditions, by exploiting diverse parts of the planet on a seasonal basis. However, migration also poses considerable risks and challenges for animals, often requiring trade-offs among physiological and behavioral processes. The collection of papers in this Research Topic shows how technological and analytical advancements permit the study of migratory strategies and physiological mechanisms in increasing detail. Importantly, many studies are now possible directly in the field or during short-term captivity, which eliminates many biases related to long-term captive experiments. Current rapid advances in tracking technologies should further accelerate our possibilities (Jetz et al., 2022). We anticipate a vibrant future for research on the ecophysiological adaptations associated with migration, and hope this Research Topic inspires future studies to elucidate the mechanisms of physiological adaptations, particularly in non-avian species and across multiple physiological systems. Such studies are also crucial to understand the potential role of physiological flexibility in responses to anthropogenic disturbances and rapidly changing environmental conditions under global change.

Author contributions

All authors contributed equally to drafting and editing of the present article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Flexibility and Control of Circadian Activity, Migratory Restlessness and Fueling in Two Songbird Migrants

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Juvenile songbirds rely on an endogenous program, encoding direction, distance, fueling, and timing of migration. Migratory distance is species-specific, expressed as a period of migratory restlessness, for which the length is correlated with distance, while fueling is modified to meet anticipated flight distances controlled by geomagnetic cues and amount of day-light available for foraging. How daylength affect onset and level of migratory activity and fueling decisions in wild birds have so far received limited attention. Here we study how photoperiod controls onset, level and extent of autumn migratory activity and fueling in juvenile diurnally migrating dunnocks, and nocturnally migrating European robins by experimentally increasing daylength. For both species, we kept a control group indoors at the location of capture in southern Sweden exposed to the natural photoperiod, and an experimental group with increased and advanced photoperiod by 2 h in the morning. Dunnocks initiated migratory activity at sunrise (or artificial sunrise) in both groups, demonstrating a highly responsive and flexible component for the onset of migration triggered by light. Experimental robins anticipated the end of nocturnal migratory activity predicting the earlier sunrise immediately after the time-shift and expressed this behavior already under darkness, supporting a fast-resetting mechanism to the new diel period. Timing of end of morning activity was not affected by the earlier sunrise in both species, suggesting a fixed endogenous control that persisted throughout the 13-day study period. Experimental dunnocks expressed higher overall activity and lower fuel loads than controls, while robins did not change their overall activity and fuel load in response to the shifted and increased photoperiod. These results reveal important adaptations for circadian timekeeping including both a flexible onset open to fast modifications and a more rigid end, with differential effects by the treatment on migratory activity and fueling in the two species.

Keywords: circadian rhythm, daylength, endogenous migration program, photoperiod, migration

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INTRODUCTION

Migratory naïve songbirds rely on an endogenous migration program guiding them from the sites where they are born to their wintering areas (Gwinner, 1975, 1996; Berthold, 1996). The genetic program encode time, distance, direction, and fueling in preparation of migration flights that enable birds to follow population-specific routes (e.g., Berthold and Querner, 1981; Helbig et al., 1989;

Berthold, 1996; Helbig, 1996, 2003; Pulido, 2007; Liedvogel et al., 2011). Additional phenotypical adaptations are needed to enable successful long-distance migrations, including morphology, physiology, and behavior (Åkesson and Hedenström, 2007). Several of those adaptations are tightly connected to the endogenous migration program (Gwinner, 1975, 1996; Åkesson et al., 2017) and the ability to navigate (e.g., Emlen, 1975; Able, 1980; Åkesson et al., 2014), while others are related to disposition of time between fueling and flight (Hedenström and Alerstam, 1997).

Both timing and length of the autumn migratory restlessness period have been shown to be correlated with migratory distance in songbirds (Berthold, 1973) and contribute to the individual variation and flexibility in migratory traits observed in birds (Åkesson and Helm, 2020). The periods of controlled migratory restlessness in individual birds are regulated by two environmental cycles, extending over a day or a full year (Aschoff, 1960; Gwinner, 1975, 1996; Gwinner and Brandstätter, 2001). The annual migratory restlessness period is controlled by an endogenous oscillator with external diel input, which in absence of seasonal variations has been shown to free run for several years (Gwinner, 1986). The circadian oscillator is dependent on control mechanisms from the day and night cycle, including variations of light levels (e.g., Aschoff, 1960; Daan, 1976; Gwinner, 1996). The complex circadian pace-making system in birds include three main components (i.e., encephalic photoreceptors, pineal gland, and retina), shown to enable coordination of time over annual and circadian cycles (Gwinner and Brandstätter, 2001).

The migration process involves periods of fueling and flight, which has been predicted to approach a fueling: flight ratio of 7:1 (Hedenström and Alerstam, 1997). Endogenous control of fueling rate and mass increase have been shown to be timed and triggered by geomagnetic information to increase in front of predicted barriers (Fransson et al., 2001), and to be regulated by daylength (Kvist and Lindström, 2000). Migratory restlessness and compass orientation have extensively been investigated in caged songbirds (e.g., Gwinner, 1975, 1986; Åkesson, 1994; Bäckman et al., 1997; Muheim et al., 2006; Helm et al., 2009; Ilieva et al., 2018). However, the combination of recording fueling, and migratory restlessness in the same experimental setup, has so far been limited (Bulte et al., 2017; Ilieva et al., 2018) as well as studies of how daylength affects migration behavior including timing of departure, activity pattern, and fueling (Kvist and Lindström, 2000).

To investigate if diel schedule has any effect on the timing and level of migratory activity, and fueling in juvenile songbirds, we designed an experiment where we experimentally increased the daylength by 2 h in the morning (i.e., prolonged photoperiod shifted forward; Müller et al., 2018b) and kept the geomagnetic parameters at the local site. By this procedure, we could separate the anticipated responses relative to the diel cycle from the geomagnetic cues (i.e., field intensity, angle of inclination) providing latitudinal information (Skiles, 1985). This was important since geomagnetic information have been shown to affect both migratory activity (Ilieva et al., 2018) and fueling (Fransson et al., 2001; Boström et al., 2010) in songbirds. We compared the behavior of the experimental group with control birds kept in the local photoperiod of southern Sweden in autumn. We studied two species of temperate zone passerine migrants captured in autumn on passage to their wintering areas in southwestern Europe, the dunnock, *Prunella modularis* and the European robin, *Erithacus rubecula* (Zink, 1975) (hereafter robin). Dunnocks migrate primarily at daytime (Dorka, 1966; Ilieva et al., 2018; Michalik et al., 2020), while the European robin is a strictly nocturnal migrant (Cramp, 1988; Åkesson et al., 2015). The daylength manipulation was substantial (2 h), and therefore out of range from what the species' normally meet on a daily basis during natural migrations, and in a direction that would be expected during spring rather than during autumn. However, since the period of the increase was abrupt and with a fixed duration (not gradual) it is unlikely that birds will interpret this change as springtime. The length of the light manipulation was selected because we wanted to investigate how the birds reacted to an earlier onset of the day and at the same time reliably measure the time needed for adjusting the clock to a new time. We were interested to investigate if the birds would shift the onset of activity and if the length of the period of migratory restlessness would stay unaffected or change in response to the manipulation. The increase in photoperiod by 2 h correspond to a 1 month shift back in time, or a geographical shift to the south of the expected wintering area in late autumn, where the experimentally increased daylength would occur (**Supplementary Figure 1**). We expected the two migrants may respond differently to the treatment, since they predominantly migrate at day (dunnock) and at night (robin). The advanced and increased photoperiod was used to investigate if the dunnock responded to the increased daylength by shifting the (fixed) period of migration restlessness in relation to the new sunrise time, or if the sunrise may result in an extended period of expressed migratory activity. For the nocturnally migrating robin we instead expected the earlier sunrise, and thus increased day-length, not to affect the start of migratory restlessness, but rather result in a shorter period of nocturnal restlessness (or no change in length) and an increased fueling rate in response to the increased period available for foraging (Kvist and Lindström, 2000).

We set up the following predictions:

(i) Dunnocks will show the highest activity in the morning, when they naturally migrate (Dorka, 1966; Michalik et al., 2020), and nocturnally migrating robins will be most active at night starting shortly after sunset (Bolshakov et al., 2000, 2007).

(ii) The length of the daily migratory restlessness period will be species-specific, related to the migration phenotype and time of year (Berthold, 1973). Therefore, we predicted that this period will be constant, and only the starting time will change with the earlier sunrise for dunnocks, while robins will not change starting time, but shorten the period of nocturnal restlessness as the migration period at night becomes shorter after light manipulation.

(iii) As an alternative to (ii), we predicted that if the sunrise is an important trigger for the onset of migration independent of a circadian time-keeper regulating the circadian rhythm and the end of the period is set relative to an endogenous dial time sense unaffected by the treatment, dunnocks would respond to the earlier sunrise by extending the period of migratory restlessness, while robins instead should be unaffected by the start and end the restlessness at the same time as controls.

(iv) Fueling rate will be negatively related to level of expressed migratory activity in both species and daylight available for foraging. With an increased daylength, longer diurnal foraging times may thus potentially increased fueling rates in both species (Kvist and Lindström, 2000), but if the daylight is primarily used for migration activity the fueling rate may instead decrease in response to the increased level of overall activity. This may lead to differences in fueling rates between the diurnal and nocturnal migrant.

MATERIALS AND METHODS

We used two species of temperate zone passerine migrants, the dunnock and the European robin, breeding in large parts of Sweden (Fransson, and Hall-Karlsson, 2008), where different populations are exposed to largely different photoperiods in summer depending on breeding latitude. Dunnock is a diurnal migrant (Dorka, 1966; Michalik et al., 2020) passing through southern Sweden on autumn migration in September and October. Swedish dunnocks migrate to wintering areas in southern France (Zink, 1975; Fransson, and Hall-Karlsson, 2008). The European robin is a nocturnal migrant, captured in large numbers on autumn migration passage in southern Sweden, migrating to wintering areas from Germany and Denmark in the north to North Africa in the south (Zink, 1975; Fransson, and Hall-Karlsson, 2008).

First-year migratory European robins ($n = 24$) and dunnocks ($n = 24$) were captured with mist-nets at stopover sites near Stensoffa Ecological Field Station (55°41′N 13°26′E) in southern Sweden on 23–24th September and 6–10th October 2018, respectively. We kept the birds indoors in individual cages for a few days (maximum 5 days) until they were all simultaneously moved to the experimental facility (on 26th September for robins and 11th October for dunnocks) when the experiments started. In the individual cages, the birds were provided with food and water *ad libitum*.

The experiments were performed in the experimental facilities at the Lund University Stensoffa Ecological Field Station in southwest Sweden (Ilieva et al., 2016). The facility is composed of six wooden houses built in non-magnetic material placed a 5–10 m apart. Each house accommodates four birds in separate circular cages ($n = 24$ birds in total). For each species, we used three of the houses as control and three houses for altering the photoperiod. All houses have a semi-transparent roof that provided natural light conditions (transmission spectrum visible in **Supplementary Figure 2**). The daylength was altered adding a LED light source (Lumak Pro; 8,000 lm luminous flux) with daylight color temperature (emitting spectrum in **Supplementary Figure 2**) in each house that was automatically switched on and off by an electronic timer. The light source was mounted pointing toward the translucent plastic roof of the house, hence, providing indirect illumination to the cages with similar diffuse light as the incoming natural light during daytime. The LED lights were switched on at 07:00 (local sunrise time) in the control houses and 2 h earlier in the treatment houses (05:00) resulting in 2 h longer days for the experimental birds. Light irradiance provided by the lamps was measured on top of the cages with an electronic radiometer (IL 1400A, International Light Technologies, Inc., United States) to be around 0.3 mW/cm^2 when complete dark outside and comparable with the light intensity measured soon after sunrise in the houses. The lights were automatically switched off in all houses at 10:00 (local time), when the natural light irradiance was above 4 mW/cm^2 (measured under different weather conditions) and the contribution due to the LED lights to the internal luminosity of the houses was not measurable anymore. We introduced the birds into the cages in the early afternoon to familiarize with the new environment and kept them in the cages during 14 days in total.

The cages were designed to electronically measure the weight of the individual birds while sitting on the perch positioned in the middle of the cage (for more details on setup see, Ilieva et al., 2016), and to let an operator provide food and water from the bottom of the cage. With these procedures, the captive birds were not able to see the operator during the weighting and while providing water and food (once a day at 12:00 local time) reducing potential stress-related effects on the birds.

In each house, a network camera was filming all four cages from above and continuously recording videos on external storing devices. Videos were then used to extract the detailed birds' activity using the computer vision procedure detailed in Ilieva et al. (2018). Briefly, the algorithm automatically detected and tracked the motion of each bird individually and measured the fraction of time that the birds were in flying mode (i.e., excluding any fluttering, jumping or walking) in 20-min intervals. Raw data from the current experiments and extracted by the above procedure are available as **Supplementary Appendix 1**, and summarized in the actogram in **Figure 1**. The daily activity recorded for the individual bird was measured as the average number of 20 min active intervals (i.e., intervals when the bird was flying for more than 1 min; Ilieva et al., 2018), and presented for the full experimental day (from 12:00 to 12:00 of the next day, local time), but also divided into first half of the day (from 00:00 to 12:00) and second half of the day (from 12:00 to 00:00) periods (**Figure 2**). In addition, we performed statistical tests in intervals before the sunrise time (either at natural or experimentally manipulated time) to investigate whether nocturnal activity in European robins was affected by the expected sunrise time. We finally tested both species for difference in "departure" and "arrival" times calculated relative to the overall diurnal activity for dunnocks (between midnight and feeding time: 00:00–12:00, local time) and nocturnal activity for robins (between sunset and sunrise: 19:00–07:00). The departure (i.e., start of migratory activity) was defined as the time at which each individual reached the 10th percentile of the cumulative activity in the considered migratory period that has been demonstrated to be an accurate predictor by Schmaljohann et al. (2015). Similarly, the arrival (i.e., end of migratory activity) was defined as the time at which the individual reached the 90th percentile of the cumulative activity, that is, when the bird is close to landing and possibly searching for shelter or a foraging ground.

All the plots and statistical tests were done in the software R version 3.6.1 (R Core Team, 2019). One-way and repeated-measures ANOVA were used for testing differences in activity, food intake, fuel load and arrival/departure time between the treatment and control groups of each species.

RESULTS

The actograms show clear differences in activity patterns between dunnocks and robins (**Figure 1**), with the highest activity in the first half of the day starting at sunrise for dunnocks, and a prolonged period with elevated level of activity during the entire night for robins. The actograms also show a strong effect of the treatment on the timing of activity in both species.

Dunnocks showed no activity at night and initiated their migratory activity at sunrise (**Figure 1**). Thereafter, the activity continued for several hours, and ended before noon. The start of activity coincided with local sunrise time for the control group exposed to the local diel cycle and was initiated 2 h before for the experimental group (a time corresponding to the apparent sunrise time in this treatment group; **Figure 1**). Dunnocks in the local photoperiod were active for the rest of the day and expressed a peak of increased activity just before sunset (**Figure 1**). The highest level of activity in dunnocks was expressed in the first half of the day, starting at sunrise and ending before noon (**Figure 1**). The experimental dunnocks expressed a similar temporal pattern of activity throughout the day, but with an extended morning activity period starting at the experimentally advanced sunrise and ending at the normal time before noon. The morning activity (higher) is primarily associated with migration, while afternoon activity (lower) is associated with foraging in dunnocks.

Robins expressed a short peak of activity just before sunset, which for this nocturnal migratory species is expected to be associated with preparations for the migratory departure. Immediately after this peak of activity at sunset, the robins showed a restricted period of lower activity corresponding to a "quiescence period," before the elevated nocturnal restlessness period started. Robins were active for the entire night, but with a reduced activity level, and showed an increased activity ending with a peak at sunrise (**Figure 1**). The timing of the peak of morning activity for the robins, probably associated with the end of the daily migratory activity and search for shelter, was affected by the treatment. That is, for the control group the peak of activity was at local sunrise time (07:00), whereas, for the experimental group the peak of activity occurred almost 2 h earlier around 05:00 when lamps were turned on for the experimental group (**Figure 1**; see also below). Both robins held in the local and extended photoperiod continued to be active during the mornings, and showed relatively limited activity during the afternoons (**Figure 1**).

To investigate whether there were differences in restlessness between groups, we analyzed the duration of activity (i.e., number of active intervals), and we found that dunnocks exposed to experimentally increased photoperiod were more active than the control group (**Figure 2**). The difference was significant during mornings (repeated measures ANOVA, days 1–13: $F_{1,22} = 4.394$, $p = 0.048$), but not during evenings ($F_{1,22} = 2.239$, $p = 0.149$). The higher activity during the morning, is a consequence of the fact that dunnocks in the daylength treatment group started their migratory activity earlier (see above) but continued to be active until around 10:00 which is similar to the time when migratory activity ceased in the control group (**Figure 1**; see also below).

Robins, on the other hand, were not affected in their overall activity by the increased photoperiod with earlier sunrise and did not change activity pattern ($p > 0.486$ for all time periods; **Figure 2**). However, we investigated if the peak activity at sunrise for robins (see above) was only affected by an external factor (i.e., the earlier sunrise of the treatment) or by an internal factor (i.e., birds can predict time of sunrise with their circadian clock). We compared the activity of the control group with the experimental group during 5 h before the sunrise, either at natural sunrise timing (07:00) or at the artificial sunrise time (05:00). The activity of robins 5 h before the natural sunrise (02:00–07:00) was not affected by treatment (repeated measures ANOVA, days 1–13: $F_{1,22} = 1.515$, $p = 0.231$). However, the activity 5 h before the artificial sunrise (00:00–05:00) showed that the experimental group was significantly more active than the control group ($F_{1,22} = 8.887$, $p = 0.007$), with higher level of activity for the experimental group. The latter difference was measured before any light was yet present in the houses. We did not find this response in the dunnocks relative to the earlier sunrise, as they expressed no activity at night (**Figure 1**).

As predicted by the actogram in **Figure 1**, we found a strong effect on time for onset of migratory activity between the control and the experimental group in dunnocks (repeated measures ANOVA, days 1–13: $F_{1,22} = 106.4$, $p < 0.001$) with the experimental group starting to express migratory restlessness earlier following the artificial sunrise (**Figure 3**). We did, however, not find any time difference in the end of restlessness for the dunnocks ($F_{1,22} = 2.04$, $p = 0.167$). Furthermore, we did not find any difference in start of migration activity between the control and the experimental group in robins ($F_{1,22} = 0.005$, $p = 0.945$; **Figure 3**), but we did find that the end of activity for the experimental group was consistently earlier than the control group ($F_{1,22} = 47.33$, $p < 0.001$), and this difference lasted during the entire experimental period (**Figure 3**).

We also measured how body mass, foraging activity and fueling varied across the experimental period, as well as between the experimental groups for the two species. There were no initial body mass differences between the control and experimental groups for both dunnocks (control: mean ± SD: 21.8 ± 3.0 g; treatment: 21.4 ± 1.6 g; one-way ANOVA: $F_{1,22} = 0.139$, $p = 0.713$) and robins (control: 16.9 ± 0.9 g; treatment: 17.0 ± 1.3 g; $F_{1,22} = 0.096$, $p = 0.759$), nor was there a significant difference between groups in food intake throughout the experiment (repeated measures ANOVA, days 1–13: dunnocks: $F_{1,22} = 2.488$, $p = 0.129$; robins: $F_{1,22} = 0.064$, $p = 0.802$; **Figure 4**). Both species showed a tendency to increase body mass during the experimental period (**Figure 4**). However,

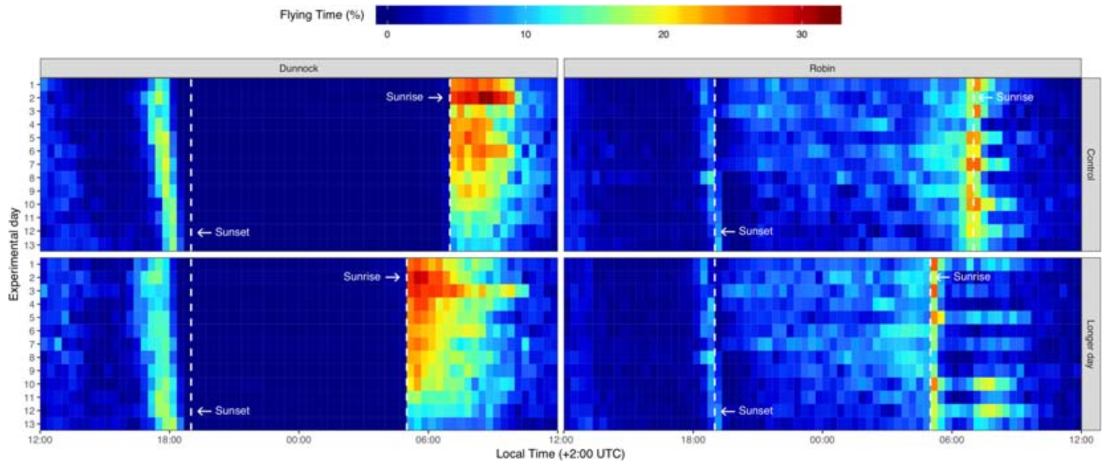

FIGURE 1 | Actogram of first-year migratory dunnocks (*Prunella modularis*) and European robins (*Erithacus rubecula*) monitored under local daylength timing (Control), and experimentally 2 h advanced early sunrise (Longer day). Each color-coded horizontal line shows the mean time spent in flying mode by 12 birds per group ($n = 24$ for each species) from 12:00 of each day to 12:00 of the successive day. Dashed vertical lines indicate the time of sunset (19:00 for all groups), as well as sunrise at local time (07:00 for the Control groups) and 2 h earlier (05:00 for the Longer day groups).

the fuel load (i.e., fraction of body mass increase relative to individual's lean body mass) for dunnocks, was significantly lower for the daylength treatment group as compared to the control group (repeated measures ANOVA, days 1–13: $F_{1,22} = 5.806$, $p = 0.025$; **Figure 4**), but there was no difference in fuel load between the two groups for robins ($F_{1,22} = 0.029$, $p = 0.866$; **Figure 4**).

DISCUSSION

Dunnocks

The dunnocks expressed the highest activity in the early morning, as confirmed in our previous study (Ilieva et al., 2018), which corresponds to the time when dunnocks normally migrate (Dorka, 1966; Michalik et al., 2020). We found that the experimental juvenile dunnocks responded to our earlier onset of sunrise (increased photoperiod) experiment, by increasing the period of diurnal migratory activity, in contrast to what has been proposed for diurnal animals (Daan, 1976). This change occurred instantly and was prolonged during the complete study period lasting 13 days. Dunnocks in the local photoperiod further expressed a peak of increased activity just before sunset, which presumably involves foraging and movements to find shelter for resting during the upcoming night (Ilieva et al., 2018). At the same time, the dunnocks exposed to an increased photoperiod reduced their fuel load, which likely was a result of the increased activity.

Our results show that the time for initiation of migratory activity was flexible and possible to experimentally advance in dunnocks. The ending of the diurnal migratory activity, however, occurred at a similar time for both the experimental group and controls ending just before noon (ca. 10:00–11:00 local time; **Figures 1**, **3**), suggesting rigid endogenous control. It, thus, seems that in diurnally migrating dunnocks the sunrise time and associated change of light level dictates the start of the migratory activity (functioning as an external trigger; DeCoursey, 1960, 1961), and an internal circadian clock dictates when the migratory activity ends rather than an inherited period length of activity. The timing of the end of activity, furthermore, did not seem to be affected by the experimentally prolonged photoperiod over time, but rather stayed constant over the 13-day study period. These results provide support that the circadian timekeeper in migratory dunnocks contain both flexible (start) and rigid (end) parts, not affected by changes of photoperiod. Inherited migration programs of diurnally migrating birds have received limited attention in experimental work so far (Åkesson and Helm, 2020). Our study of dunnocks, thus, provide new information on how photoperiodic changes affect onset of migratory activity, the length of it, and how the end is kept constant despite an increased photoperiod, with an increased level of activity and reduced fueling as a result.

A flexible light-dependent control mechanism triggering migratory activity (DeCoursey, 1960, 1961), will enable adaptive responses to local photoperiods and weather conditions during migrations (e.g., Åkesson et al., 2001, 2002; Sjöberg et al., 2015, 2017; Müller et al., 2016), but may also be an important regulator of speed of migration (Hedenström and Alerstam, 1998; cf. Lindström et al., 2019) by controlling fueling rates (Kvist and Lindström, 2000) and energy consumed. Such a light-dependent triggering mechanism affecting migratory activity may, however, also be vulnerable to human impact from light pollution as reported for nocturnal bird migrants (e.g., La Sorte et al., 2017; Horton et al., 2019), with elevated risk of mortality and fatal conservation consequences (Van Doren et al., 2017; McLaren et al., 2018). The influence of artificial light on ecosystems are multiple and widespread (Gaston et al., 2013) and will predictably be strong for diurnal bird migrants as shown in our experiments with migratory dunnocks, leading to extended

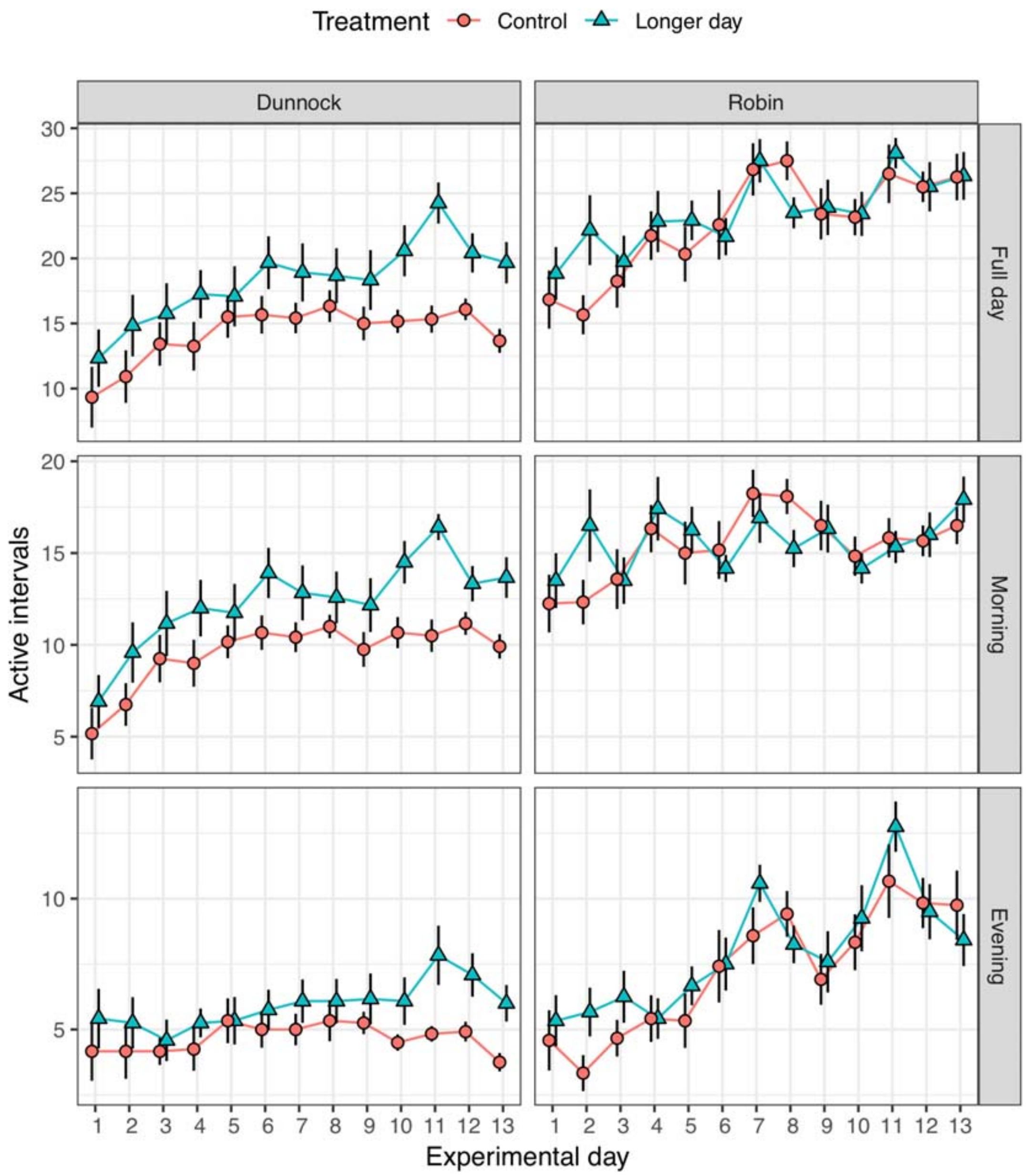

FIGURE 2 | Average (±SE) daily activity of first-year migratory dunnocks (*Prunella modularis*) and European robins (*Erithacus rubecula*) monitored under local daylength timing (Control) and early sunrise (Longer day) conditions ($n = 12$ for each treatment group; $n = 24$ for each species). The daily activity was measured as the average number of 20-min active intervals (i.e., intervals when birds were flying for more than 1 min) presented for the full day, and divided in morning and evening periods.

activity periods, and reduced fueling rates with likely negative fitness consequences. Therefore, a more extensive study of this problem with respect to artificial lights in natural environments would be of great interest for the future.

European Robins

The European robins in our experiments showed strictly nocturnal migratory activity, which has been observed in previous experiments in our facility (Bianco et al., 2019), and reduced activity at daytime (mid-day and afternoon). The robins also expressed an increased peak of activity associated with the time of sunset, as well as with the early morning hours (**Figure 1**). The sunset peak of activity was followed by a period of lower activity, i.e., a quiescence period, before the nocturnal migration restlessness was initiated (**Figure 1**), and this period is expected to be associated with preparations for migration departure observed in birds both in captivity (e.g., Watts et al., 2017) and during natural migrations (e.g., Hebrard, 1971; Åkesson et al., 1996a; Bolshakov et al., 2007; Schofield et al., 2018). The time for nocturnal migration departure shows substantial

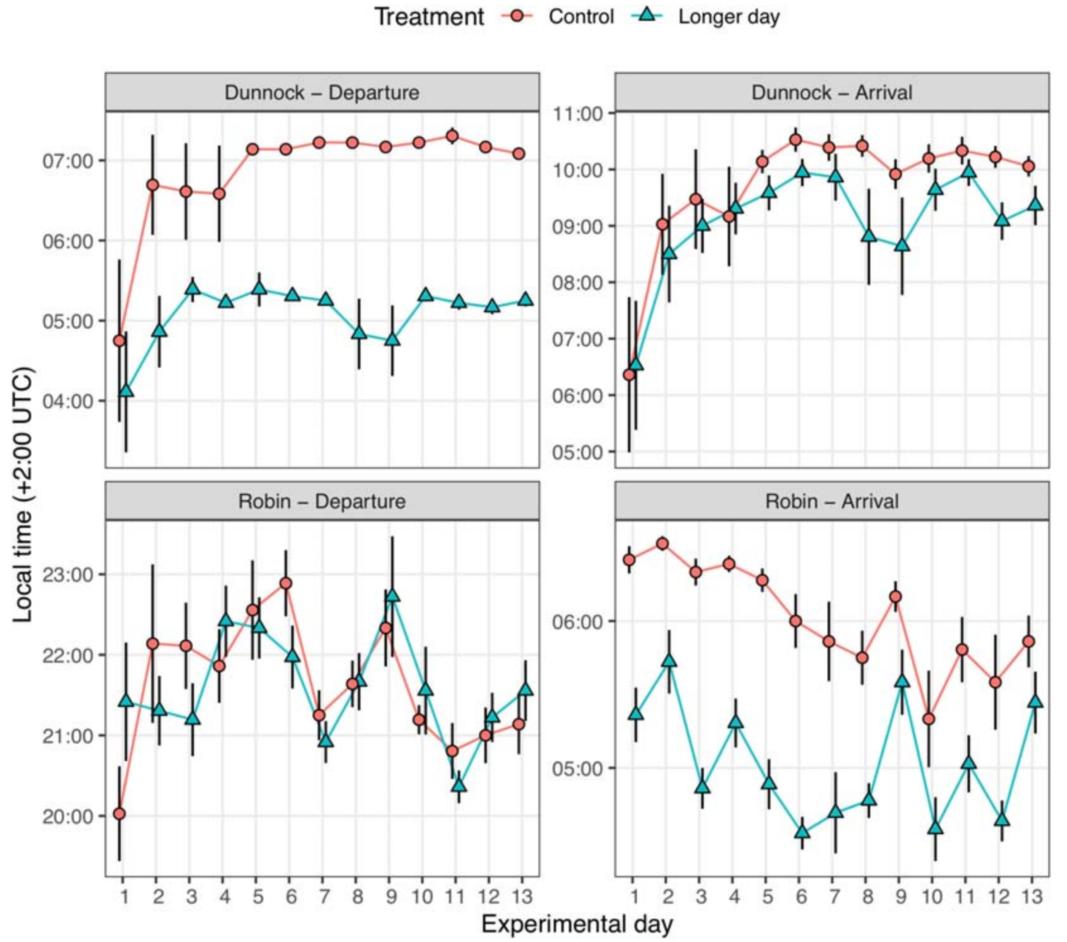

FIGURE 3 | Time (±SE) of migratory departure and arrival for first-year migratory dunnocks (*Prunella modularis*) and European robins (*Erithacus rubecula*) monitored under local daylength timing (Control) and 2 h experimentally advanced early sunrise (Longer day) conditions ($n = 12$ for each treatment group; $n = 24$ for each species). For information on categories, see main text.

inter-individual variation for different bird species (Hebrard, 1971; Åkesson et al., 1996a; Bolshakov et al., 2007; Schofield et al., 2018), where several ecological factors such as internal state, stopover time, and weather likely have important influences on the timing of migration (e.g., Åkesson and Hedenström, 2000; Åkesson et al., 2001, 2002; Bolshakov et al., 2007; Sjöberg et al., 2015, 2017; Müller et al., 2016). Migratory intensity, recorded in diurnal and nocturnal free-flying songbird migrants, follows a predictable temporal pattern relative to sunset and sunrise with increased migration traffic rate starting at civil twilight at dusk, with the peak activity recorded 2–4 h thereafter, and again starting 1 h before civil twilight at dawn with continued activity after (Zehnder et al., 2001; Åkesson et al., 2001). European robins may initiate the migration at different times of the night, and show the highest migration activity during the second half of the night as shown for tracked birds in field studies (Åkesson et al., 1996a; Bolshakov et al., 2000, Bolshakov et al., 2007). Our data from diurnally migrating dunnocks initiating their migration activity at sunrise, and nocturnally migrating robins showing the highest activity starting 1–2 h after local sunset and continuing for the rest of the night support these findings.

We found an interesting difference in response to the increased and advanced photoperiod for the robins associated with one of the two sunrise and sunset activity peaks. Robins were not affected in the start of the migration activity period by the advanced and increased day length, but they were affected in the timing of end of migration activity. Since the pre-dawn activity increased when the birds were still in the dark, it means that the experimental robins were able to predict the new sunrise time in their house immediately after the first day experiencing a shifted sunrise time. The time of sunrise was anticipated before any light was present in the houses and supports the hypothesis that the robins were able to use their circadian time sense to predict the artificial sunrise time and adjust their migratory activity accordingly. Our experiments suggest that this response was expressed instantly, involving unexpected flexibility (**Figures 1**, **3**), in line with the findings reported by DeCoursey (1960, 1961) for flying squirrels reacting to a short pulse of light by increasing activity. Thus, our

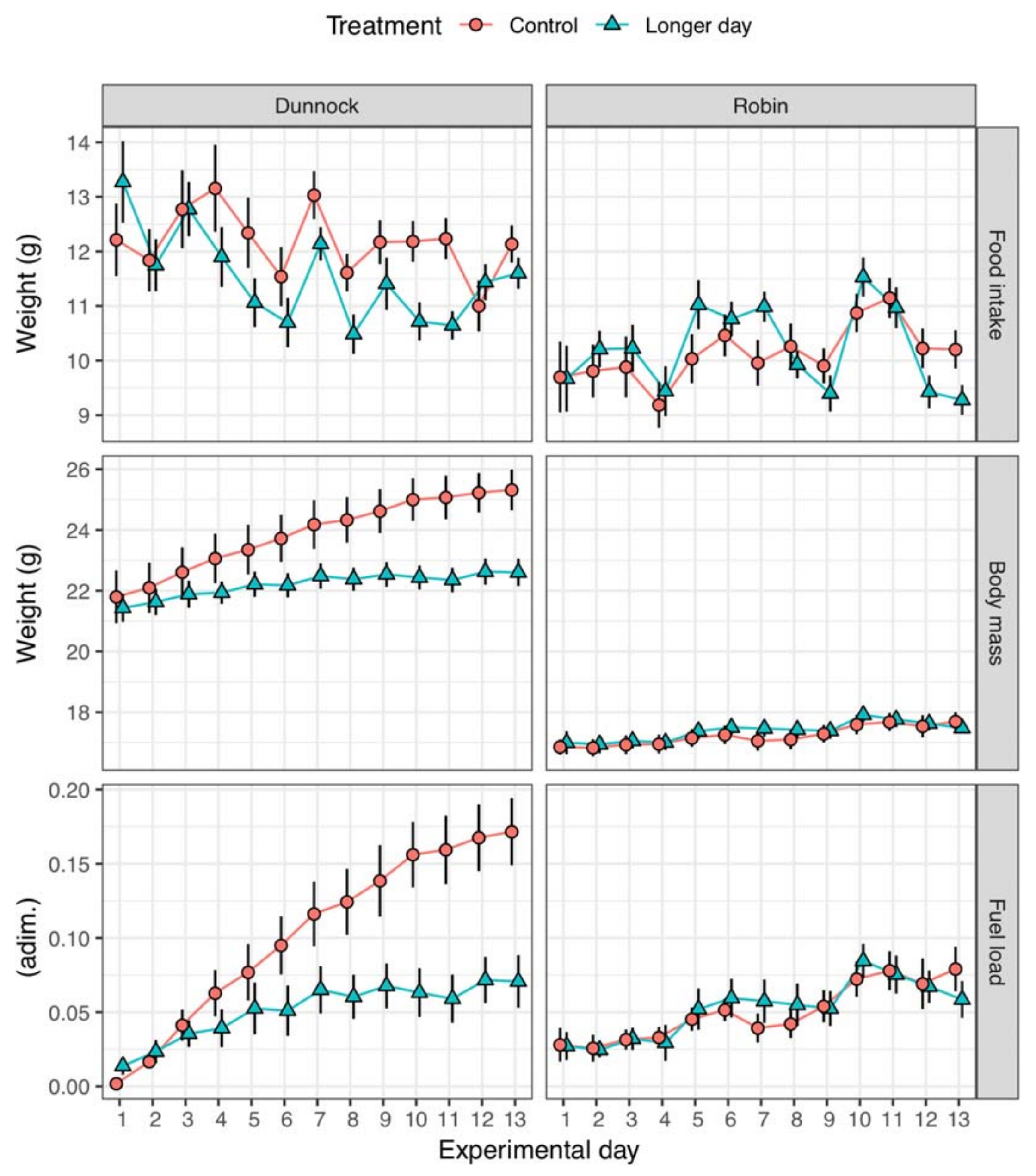

FIGURE 4 | Average (±SE) of food intake, body mass and fuel load for first-year migratory dunnocks (*Prunella modularis*) and European robins (*Erithacus rubecula*) monitored under local daylength timing (Control), and 2 h experimentally advanced early sunrise (Longer day) conditions ($n = 12$ for each treatment group; $n = 24$ for each species).

results suggest that indeed the robins were affected in their behavior by the extended morning period, not in the overall daily activity, but in the timing of their activity including the activity expressed before sunrise. This light-level-trigger associated with the night-day transition could for nocturnally migrating robins be used to decide the landing time after a nocturnal migratory flight, and the increased desire to move at daybreak may be used to visually find safe shelter, to avoid diurnal predators and search for good foraging sites (Alerstam, 1978). In coastal areas, re-orientation by nocturnal avian migrants to sheltered areas have frequently been observed in early morning hours, and may be associated with movements to inland stop-over sites (e.g., Åkesson et al., 1996b, 2001; Zehnder et al., 2002; Nilsson and Sjöberg, 2016).

Fueling and Activity

We found an overall but limited increase in mass and fueling rate in our control birds over the study period. At the same time, there was no effect on mass gain by the increased photoperiod in robins, and a very strong negative effect on fueling in dunnocks.

In the experimental dunnocks, the decreased fueling rate was associated with an increased level and prolonged period of migratory activity expressed during the day. Time of season has been shown to affect fueling decisions and mass gain in caged songbird migrants (Kullberg et al., 2003, 2007), with a higher rate in late birds. Availability of food has further been shown to affect the level of migratory activity in songbirds, resulting in reduced activity after food being presented *ad libitum* (Biebach, 1985; Gwinner et al., 1985). All our control and experimental birds were given access to water and food *ad libitum* during the study period, and still we observed an increased level of activity in dunnocks in response to the earlier sunrise, suggesting the light cue was more important as a trigger of migratory activity than food availability. It was further interesting to note that the experimental robins did not explore the increased daylength for foraging, to increase the fueling rate, as has been predicted for bird migrants (Kvist and Lindström, 2000). Instead, it seems like the fueling rate may be controlled by other factors for the robin, which may be interesting to study in the future. In line with this, Maggini and Bairlein (2010) found that different populations of Northern wheatears (*Oenanthe oenanthe*) expressed migratory activity in relation to anticipated migration distance while fueling were related to the distance of barrier crossings which differed between populations. Thus, the two components, activity and fueling, seems to be controlled by different factors in the wheatears, leading to adaptive levels of fueling during migration instead of maximizing fuel intake at any given time. One additional factor limiting fueling rate (mass gain) could, for instance, also be the risk of being taken by a predator, which would lead to reduced fueling in response to increased predation risk (Lind et al., 2010).

Photoperiodic Time-Keeping as an Adaptation to Control Migration

Our experimental treatment, increasing day length by 2 h is far off what our species are experiencing during natural migrations at this time of year, but have been used in previous experiments with songbirds (Müller et al., 2018b). Daylength may provide information on latitude, and as such potentially also anticipated migration distance, passed, and remaining. Our experiments were performed with caged songbird migrants during the autumn equinox period, when birds naturally will experience no difference in day length between latitudes at the autumn equinox (23 September), but fast changing day length near to this date, and different lengths of the twilight period depending on latitude (Daan and Aschoff, 1975). Despite this, we found very pronounced effects on timing and level of migratory activity in diurnal dunnocks, and a shift in timing-related activity peaks in nocturnal robins in response to the 2 h earlier sunrise. Since we kept the geomagnetic parameters (i.e., field intensity and angle of inclination) at local values, we can be sure that the birds responded to the daylength manipulations and not the geomagnetic field.

It is important for migratory birds to time their movements with respect to environmental conditions, geographical areas and circannual cycles, and to manage this an efficient control mechanism will be needed (Åkesson et al., 2017). Migratory birds seem to have evolved a complex navigational toolbox, shaped by generations of successful exploration of wide geographical space (Wehner, 1998). Still, several of the navigational tools used for global navigation remain unknown, and underlying sensory mechanisms and the ways they are combined even more so. Clearly space and time needs to be combined in the endogenous migration program of birds (Gwinner, 1986, 1996; Wallraff, 1990; Wehner, 1998). Latitudinal information can be provided by geophysical information from the geomagnetic field (total field intensity and angle of inclination; Skiles, 1985), and have been shown to affect fueling and migratory activity in juvenile songbirds (e.g., Fransson et al., 2001; Boström et al., 2010; Ilieva et al., 2018). However, a photoperiodic control mechanism with high adaptability to the transition between day and night may further assist birds to adjust to the local time on migration. Such mechanism may for instance be part of the re-setting of the time-compensated sun compass prior to migration flights (Alerstam and Pettersson, 1991). It may also trigger level and extent of migratory activity as suggested here, and could contribute to a photoperiodic control mechanism recording daylength that could be used to adjust the endogenous program to local cues (Müller et al., 2016). At this point, we cannot exclude that a flexible diel control mechanism may be combined with the perception of geomagnetic information to define latitude, and we suggest that this possible association should be studied in the future.

Our results have revealed some interesting differences between two passerine species migrating similar distances, along similar southwestern routes and at the same time of year but adapted to migrate at different time of day. Still, there are several aspects of their endogenous migration program that would be interesting to investigate more closely in the future, such as the effect of decreased (and increased) photoperiod on activity and fueling rate. Comparative experiments on these two and other species of songbird migrants outside of the equinox period may also be of priority since species-specific differences in circadian pace making may be expected (Gwinner and Brandstätter, 2001). It will also be interesting to study how different phenotypic components related to the endogenous migration program may be affected by an extended daylength at sunset rather than at sunrise. In such scenario, we may at first expect opposite responses between diurnal and nocturnal songbird migrants as observed here, but also potentially species- and population-specific adaptations depending on migration timing and distance (Maggini and Bairlein, 2010). We hope that future research will reveal in what aspects and level of detail migration programs and control mechanisms vary between species, and how these adaptations have evolved to cope with differences in migration routes and strategies for different bird species and populations (Müller et al., 2018a). We believe that our experimental setting applied here may be a useful approach to resolve some of these challenging and still open questions on the functional characteristics of endogenous migration programs that can be applied to birds and other animals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Malmö/Lund Ethical Committee for scientific work on animals (Dnr 5.8.18-12719/2017), the Swedish Board of Agriculture for housing facilities (Dnr 5.2.18-5398/16), and work with animals (Dnr 5.2.18-10992/18).

AUTHOR CONTRIBUTIONS

MI performed the fieldwork with support from GB and SÅ. GB analyzed the data. All authors conceived the study, discussed the outcome of the results, and wrote the first draft of the manuscript.

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frontiers in Ecology and Evolution

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Physiomorphic Transformation in Extreme Endurance Migrants: Revisiting the Case of Bar-Tailed Godwits Preparing for Trans-Pacific Flights

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In a 1998 paper entitled "Guts don't fly: small digestive organs in obese bar-tailed godwits," Piersma and Gill (1998) showed that the digestive organs were tiny and the fat loads huge in individuals suspected of embarking on a non-stop flight from Alaska to New Zealand. It was suggested that prior to migratory departure, these godwits would shrink the digestive organs used during fuel deposition and boost the size and capacity of exercise organs to optimize flight performance. Here we document the verity of the proposed physiomorphic changes by comparing organ sizes and body composition of bar-tailed godwits *Limosa lapponica baueri* collected in modesty midway during their fueling period (mid-September; fueling, $n = 7$) with the previously published data for godwits that had just departed on their trans-Pacific flight (October 19; flying, $n = 9$). Mean total body masses for the two groups were nearly identical, but nearly half of the body mass of fueling godwits consisted of water, while fat constituted over half of total body mass of flying godwits. The two groups also differed in their fat-free mass components. The heart and flight muscles were heavier in fueling godwits, but these body components constituted a relatively greater fraction of the fat-free mass in flying godwits. In contrast, organs related to digestion and homeostasis were heavier in fueling godwits, and most of these organ groups were also relatively larger in fueling godwits compared to flying godwits. These results reflect the functional importance of organ and muscle groups related to energy acquisition in fueling godwits and the consequences of flight-related exertion in flying godwits. The extreme physiomorphic changes apparently occurred over a short time window (≤1 month). We conclude that the inferences made on the basis of the 1998 paper were correct. The cues and stimuli which moderate these changes remain to be studied.

Keywords: body composition, exercise, fat stores, migration, organ, shorebird

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INTRODUCTION

The bodies of individual animals are not only in constant material and energy flux, but also constantly changing in mass and composition as individuals mature and age and make adjustments to external conditions like weather and food. Such changes may also reflect seasonally changing "goals and ambitions" – whether these relate to reproduction, molt or migration (Piersma and van Gils, 2011). The greater the variability in external demands in response to the manifold seasonal challenges, the greater the corporeal changes with respect to physiological and morphological phenotypic traits one come to expect. The impressive individual bodily changes during long-distance migration of birds discussed here, along with the fast morphological changes in snakes as they go from a fasting state to one of digesting large prey (Secor et al., 1994), have served as starting points in the field (Piersma and Lindström, 1997). To capture the simultaneous physiological (i.e., nervous, endocrine, and other biochemical states) and organ-level transformations shown by animals responding to changing context, Oudman et al. (2016) used the term "physiomorphic" as a unifying term. Among the great diversity of avian life forms, the extreme endurance migrants (Piersma, 2011; Conklin et al., 2017) would be expected to embody the most extensive physiomorphic changes.

Here we describe the physiomorphic restructuring in extreme endurance migrant birds during their transition from fueling to flight. In a 1998 paper entitled "Guts don't fly: small digestive organs in obese bar-tailed godwits," Piersma and Gill (1998) showed that in individual bar-tailed godwits *Limosa lapponica baueri* (Naumann, 1836) suspected of embarking on a non-stop flight from Alaska to New Zealand, the digestive organs were tiny and the fat load huge. Lacking a comparison with fueling birds, it was suggested that shortly prior to migratory departure, godwits would shrink the digestive organs used during fuel deposition and boost the size and capacity of exercise organs to optimize flight performance. Such patterns have meanwhile been established in other shorebird migration systems (Piersma et al., 1999; Landys-Ciannelli et al., 2003; Hua et al., 2013). In this contribution, we present the compositional analysis of a new sample of fueling godwits in comparison with the published in-flight sample, which now substantiates the suggestions made by Piersma and Gill (1998) about organ-restructuring for migration.

STUDY SYSTEM

Bar-tailed godwits of the *baueri* subspecies breed in western Alaska and migrate south on a non-stop 10-day flight across the Pacific Ocean and spend the northern winter in eastern Australia and New Zealand; returning in spring to the Alaska breeding grounds in two long endurance flights interrupted by a refueling period in the Yellow Sea (Gill et al., 2009; Conklin et al., 2011; Battley et al., 2012). Before their southward migration, bar-tailed godwits stage at sites in Alaska along the coasts of the Yukon–Kuskokwim Delta and Alaska Peninsula estuaries from mid-July through October (Gill et al., 1981; Gill and Handel, 1990; Gill and McCaffery, 1999) and time their departure with favorable wind conditions (see Gill et al., 2009, 2014 for details). The initiation of southbound migration from Alaska to Australia and New Zealand varies annually but typically commences in late August or early September (McCaffery et al., 2006; McCaffery and Gill, 2020). Most adult godwits precede juveniles, such that adults typically begin arriving at non-breeding destinations in Australia and New Zealand in early or mid-September, while most juveniles do not arrive prior to October (T. Habraken and A. Riegen personal communication).

MATERIALS AND METHODS

The samples used in our comparisons derive from two sites on the Alaska Peninsula. The first sample consists of nine juveniles (all males) salvaged after colliding with a large (12-m diameter) radar dome near Cold Bay, Alaska, at 23:30 h on October 19, 1987. Cold Bay is located near the tip of the ∼500-km-long Alaska Peninsula. Given the location (3–5 km inland of nearest intertidal habitat), time of day and season, and prevailing weather when the dome-strike occurred, it is believed that this sample represent godwits actively embarking upon southbound migration; see Piersma and Gill (1998) for details. The second sample of bar-tailed godwits (also all juveniles; two females and five males) was collected September 17–18, 2005 at Egegik Bay, a sampling period timed 2–4 weeks prior to the expected onset of southbound migration for juveniles. Egegik Bay is located ∼450 km northeast of Cold Bay, and has been identified as an important site for bar-tailed godwits during fall (boreal) migration (Gill and McCaffery, 1999). The juvenile godwits treated here thus represent a sample of birds (Egegik Bay) in the fueling annual-cycle stage of their migration, characterized by hyperphagia and possibly still some active body growth (Battley and Conklin, 2010), compared with a sample (Cold Bay) in the flight stage of their long-distance migration across the Pacific. Typically, bar-tailed godwit eggs hatch in the second week of June through the first week of July in Alaska (McCaffery and Gill, 2020). Assuming an average hatch date of June 21 for the birds in our sample, the Egegik Bay godwits would thus be approximately 3 months old when collected and the Cold Bay godwits approximately 4 months old.

Compositional Analysis

The specimens from Cold Bay and Egegik Bay were preserved and processed similarly. Two of the nine godwits from Cold Bay died upon impact with the radar dome, while the remaining seven surviving godwits were euthanized by chest compression 10 h after impact in view of the gravity of their injuries. Subsequent analysis determined no difference between the body composition of godwits that died upon impact and those that were euthanized after 10 h (see Piersma and Gill, 1998 for details). At Egegik Bay, godwits were collected by REG by shooting. Birds not killed instantly were immediately euthanized by chest compression. Godwits from both sites were weighed within an hour after death [using a spring scale (±5 g) and electronic balance (±1 g) for the flighted birds from Cold Bay and the fueling birds from Egegik Bay, respectively], and measurements of the exposed culmen and diagonal tarsus were collected [both using calipers (±0.1 mm)]) [note that we also measured wing chord (±1 mm), but as the measurements of wing length of the two samples were based on different methods, we refrained from using this measure of body size]. After the collection of external measurements, godwits from both sites were sealed in airtight plastic bags and frozen at −20°C.

Compositional analysis for both groups of godwits was performed similarly and consisted of dissecting birds into relevant organ and muscle groups; see Piersma and Gill (1998) and Landys-Ciannelli et al. (2003) for details related to specimens from Cold Bay and Egegik Bay, respectively. Birds from both sites were first plucked of feathers. Next, for birds from Cold Bay, the right flight muscle (including the pectoralis and supracoracoideus muscles; see Piersma and Dietz, 2007), heart, gizzard, esophagus and proventriculus, liver, left kidney, and gut were removed and weighed wet to the nearest 0.01 g. The length of the gut was measured to the nearest 1.0 mm, then gently flushed with water and reweighed empty; the gizzard and proventriculus were similarly emptied and reweighed.

To determine the compositional analysis of godwits from Cold Bay, a ground homogenate was made for each bird by passing the entire carcass (minus contents from the gut, gizzard, and proventriculus) multiple times through a food grinder (see Brown and Fredrickson, 1986). From each godwit, a random sample of the homogenate (∼10% of bird body weight) was collected and dried in a vacuum at 100°C for 15 h to determine water content. The lipid content of each sample was determined using Soxhlet extraction with petroleum ether following procedures described by AOAC (1984). Results of these analyses were expressed as proportions of fresh body mass from which we determined total fat, water, and fat-free dry masses for each specimen.

For godwit specimens from Egegik Bay, the heart, stomach (gizzard and proventriculus), liver, both kidneys, gut, and

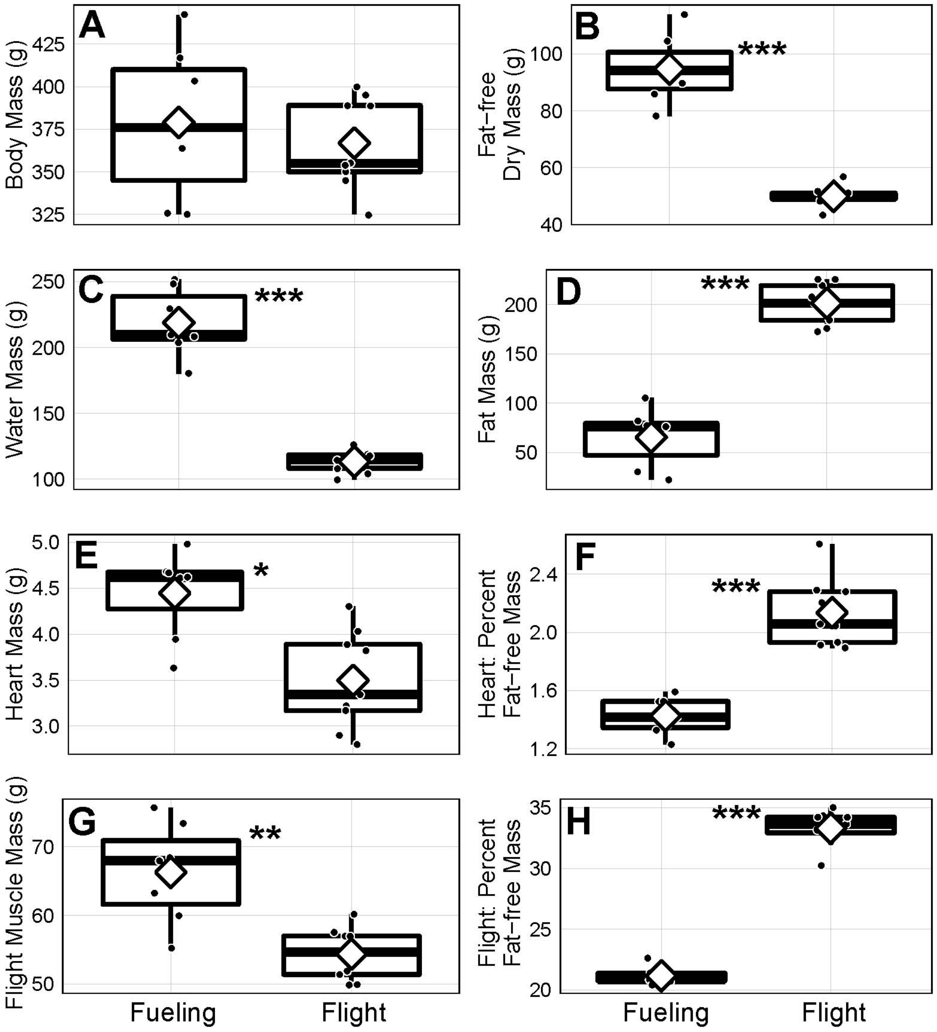

FIGURE 1 | Mass **(A–E,G)** and mass relative to fat-free mass **(F,H)** of body components of juvenile bar-tailed godwits sampled in Alaska, United States, during the fueling (*n* = 2 females and 5 males) and flight (*n* = 9 males) annual-cycle stages of their first southward trans-Pacific migration. Horizontal lines represent the median, diamonds the mean, black circles the actual values, boxes the 25th and 75th percentiles, and whiskers the range of values. Statistically significant group differences based on analysis of variance analyses controlling for effects of sex represented by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

pectoralis and supracoracoideus muscles (from both sides) were dissected from each bird. These organs and muscles were weighed wet to the nearest 0.01 g. As with the birds from Cold Bay, the length of the gut was measured to the nearest 1.0 mm, then gently flushed with water and reweighed empty. The stomach was similarly emptied and reweighed. Each of these discrete organ and muscle groups was then dried along with the carcass at 60°C for ≥24 h and reweighed. These component pieces were next individually wrapped in filter paper and placed in a Soxhlet apparatus from which lipids were extracted using petroleum ether. Samples were again dried at 60°C for ≥24 h and weighed. From each Egegik Bay specimen we subtracted the dry mass from the wet mass to determine the water mass, and the fat-free dry mass from the dry mass to determine the fat mass. The sex of all specimens was determined based on gonadal inspection during dissections.

Statistical Analyses

We summed the values for the supracoracoideus and pectoralis muscles for bar-tailed godwits from Egegik Bay to determine the mass of each bird's total flight muscle and then doubled the values of right flight muscle and left kidney from godwits from Cold Bay to standardize values between the two sample sites. For sake of comparison to previous studies (i.e., Piersma et al., 1999; Battley et al., 2000; Landys-Ciannelli et al., 2003), we summed the values of gizzard, esophagus and proventriculus from Cold Bay godwits to match the value of stomach mass from Egegik Bay godwits. Note, however, that Piersma and Gill (1998) reported values for gizzard alone.

Bar-tailed godwits exhibit sexual size dimorphism, with females averaging larger than males (Piersma and Jukema, 1990; Conklin et al., 2011). We corrected for sex-specific differences in the mass of organs and muscle groups by comparing values between the fueling birds from Egegik Bay and the flighted birds from Cold Bay using analysis of variance with sex as an additive factor. However, as we detected no significant differences in comparisons between females (n = 2) and males (n = 14) in our analyses, we combined the sexes in graphical comparisons between the two samples. As the mean body masses were nearly identical for the two sites, relative values would have been almost equivalent to what we present.

Due to unequal sample sizes in our groups, we fit analysis of variance models using type II sums of squares, and tested underlying model assumptions of normality and equality of variance using standard diagnostics (Faraway, 2002). We conducted all analyses using R software (R Core Team, 2021) and considered differences to be biologically meaningful at $\alpha = 0.05$. Summaries are presented as mean ± standard deviation unless otherwise noted.

RESULTS

There were few structural differences in body components between Egegik and Cold Bay birds. The average body mass of bar-tailed godwits fueling at Egegik Bay (379.0 ± 44.6 g, n = 7; the two females weighed 442 and 325 g, with an average of 383.5 g, similar to the five males who averaged 377.2 ± 35.5 g, range 326–417 g) was not statistically significantly different from that of birds at Cold Bay at the start of their migratory flight (366.9 ± 26.7 g, n = 9; **Figure 1A**; $F_{1,13} = 0.3$, $p = 0.62$). Likewise, the length of the exposed culmen did not differ significantly between the two groups ($F_{1,13} = 3.3$, $p = 0.09$; 80.0 ± 8.6 mm for Egegik Bay males, 102.8 ± 12.5 mm for Egegik Bay females, and 71.9 ± 6.8 mm for Cold Bay males), although the diagonal tarsus was longer in godwits from Egegik Bay (55.8 ± 3.1 mm for males, 60.0 ± 1.6 mm for females) than Cold Bay (52.9 ± 1.2 mm; $F_{1,13} = 6.4$, $p = 0.03$).

The similar body mass values stand in contrast to their composition, with the fueling birds consisting of 25.1% fat-free dry tissue, 58.0% water, and 17.0% fat, while the corresponding values for migrating birds from Cold Bay were 13.6% fat-free dry tissue, 30.9% water, and 54.8% fat (**Figure 2**). The mass of fat-free dry tissue (**Figure 1B**; $F_{1,13} = 110.5$, $p < 0.001$), water (**Figure 1C**; $F_{1,13} = 102.2$, $p < 0.001$), and fat (**Figure 1D**; $F_{1,13} = 89.7$, $p < 0.001$) all differed between the two groups. As expected (Piersma and van Brederode, 1990), water content of the fat-free mass of the two groups was similar (fueling group: 69.8%, flight group: 69.4%). In the flight group, fat-free dry mass (49.9 ± 3.5 g) was 52.7% that of the fueling group (94.7 ± 11.9 g). There was one additional source of heterogeneity, however. In the flight group from Cold Bay, the two dying on impact showed a water content of fat-free mass of 70.4%, whilst the value for the seven that were held for 10 h before euthanization was 68.0%, i.e., they lost a little water.

The proportionately low fat-free dry mass values (**Figure 2**) in the flight group resulted from reductions in the size of all measured body parts compared to those in the fueling group.

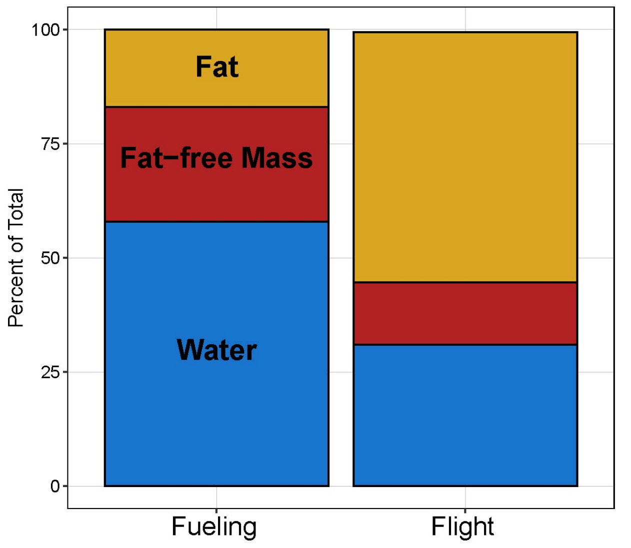

FIGURE 2 | Average composition [percent of total mass comprised by water (blue), fat-free tissues (red), and fat (yellow)] of juvenile bar-tailed godwits sampled in Alaska, United States, during the fueling (September 17–18; n = 2 females and 5 males) and flight (October 19; n = 9 males) annual-cycle stages of their first southward trans-Pacific migration.

This included the fresh mass of the primary exercise organs, the heart (**Figure 1E**; $F_{1,13}$ = 8.4, p = 0.01) and the flight muscles (**Figure 1G**; $F_{1,13}$ = 11.4, p = 0.004), which were heavier in bar-tailed godwits sampled during fueling than during flight. Nevertheless, as a fraction of the overall fat-free mass, the mass of heart (**Figure 1F**) and flight muscles (**Figure 1H**) showed proportional increases during the transition from fueling to flight ($F_{1,13}$ = 40.3, $p < 0.001$ for heart, $F_{1,13}$ = 333.5, $p < 0.001$ for flight muscle).

For the organs related to digestive and homeostatic functions, the fresh mass of liver (**Figure 3A**; $F_{1,13}$ = 128.3, $p < 0.001$), kidneys (**Figure 3C**; $F_{1,13}$ = 36.5, $p < 0.001$), stomach (**Figure 3E**; $F_{1,13}$ = 18.5, p = 0.03), and intestine (**Figure 3H**; $F_{1,13}$ = 135.4, $p < 0.001$) were all greater in fueling than in flying godwits. The length of the intestine was also longer in fueling godwits compared to flying godwits (**Figure 3G**; $F_{1,13}$ = 62.2, $p < 0.001$). However, in contrast to the sizes of heart and flight muscle, the relative masses of these organs (as a percentage of overall fat-free mass) had become smaller in flying compared with fueling godwits [**Figure 3B** ($F_{1,13}$ = 78.3, $p < 0.001$) for liver; **Figure 3D** ($F_{1,13}$ = 6.8, p = 0.02) for kidney; $F_{1,13}$ = 141.0, $p < 0.001$ for intestine]. The exception here was the mass of the stomach, which was relatively heavier in flying godwits (**Figure 3F**; $F_{1,13}$ = 4.2, p = 0.02).

DISCUSSION

Although the mean total body masses of the fueling and flying groups were similar, fat contributed a small fraction of 17% to the bodies of fueling godwits but comprised over half in the flying godwits. Interestingly, not only were the organs related to digestion and homeostasis heavier in fueling godwits, so were heart and flight muscles; although the latter two components constituted a relatively greater fraction of the fat-free mass of flying godwits. The sampling of the juvenile bar-tailed godwits occurred a month earlier at Egegik Bay than in Cold Bay. Although we do not know their precise ages, we do know that the older Cold Bay birds died in migratory flight (Piersma and Gill, 1998), whereas the birds from Egegik Bay were collected whilst foraging on intertidal flats on the known staging grounds (Gill and Handel, 1990; Gill and McCaffery, 1999). The differences in composition between the fueling and flying groups should reflect the difference in the annual-cycle stage during which they were sampled rather than differences in age.

In previous studies on body composition and organ sizes in (re-)fueling red knots *Calidris canutus* and bar-tailed godwits based on sequential sampling, the absolute sizes of flight muscles and heart always increased in the week before take-off on long-distance migratory flights (Piersma et al., 1999; Landys-Ciannelli et al., 2003; Hua et al., 2013). Preparations also included increases in hematocrit (Piersma et al., 1996; Landys-Ciannelli et al., 2002), the correlated increases in the viscosity of the blood apparently necessitating a stronger, bigger pump (i.e., the heart; Piersma et al., 1999). In the present study, the increases in relative flight muscle mass are consistent with an increasing power requirement for similar flight performance as weight load increases (Lindström et al., 2000; Dietz et al., 2007). Yet, the decreases in absolute (rather than relative) size of heart and flight muscle mass in fueling vs. flying juvenile *baueri* godwits shown here are unexpected. The relative increase in heart size may still allow adequate blood circulation due to a structurally smaller "working body" (expressed in fat-free mass), as the enormous fat load at the start of flight would be poorly vascularized. The small flight muscles relative to body mass inevitably lead to reductions in take-off speed and flight maneuverability, thus increasing predation danger in the birds that are ready to depart (Burns and Ydenberg, 2002; van den Hout et al., 2010) should aerial predators be around. As argued before (Piersma, 1998), the outcomes of these trade-offs indicate the extreme pressures on the "economic design" (Yang et al., 2013) necessitated by the trans-Pacific non-stop flights of 11,000 km or more (Gill et al., 2009; Battley et al., 2012).

Fully consistent with the earlier findings (Piersma et al., 1999; Landys-Ciannelli et al., 2003; Hua et al., 2013) were the decreases, both in absolute and relative terms, of the digestive and processing organs such as liver, kidney, stomach, and intestine (with relative stomach mass showing a small increase, **Figure 3F**). This makes sense as, during the non-stop endurance flights of more than a week, there is no need to maintain any machinery other than that for active flight, provided that the ecological conditions (food and danger from predators) at the ports of call in Australia and New Zealand are permissive to "fully depleted" arriving birds (see discussion in Piersma et al., 2021). To illuminate the ecological and physiological constraints on endurance performance, information on the "design" consequences of ecological context during arrival and physiological knowledge on the requirement to liberate amino acids from proteinaceous tissue for repair work during the non-stop flights (Battley et al., 2000; Guglielmo et al., 2001), are now in urgent need of further study. This would include answers to questions on the apparent "defense" of stomach size in the transformation to endurance flight, reminiscent of similar organ "behavior" in starving red knots (Dietz and Piersma, 2007), and the striking lack of mass loss in the stomachs of great knots *Calidris tenuirostris* during northward non-stop flights of 7,000 km from northwest Australia to the Yellow Sea when most other organs except the brain lost mass (Battley et al., 2000).

All these changes lead to individual avian bodies which in the course of 2–4 weeks do not change in mass, but strongly increase in fat load, culminating in 58% fat in the sample of bar-tailed godwits colliding in flight with the radar dome at Cold Bay (**Figure 2**). As fat has an energy density four times higher than wet protein (i.e., the building material of organs and muscles; Jenni and Jenni-Eiermann, 1998), over the period of transformation the energy content of the godwits continues to increase. Assuming that the phenology of migration was the same in both years so that a 4-week period realistically separates the two sampling events, the observed increase in fat mass from 65.5 to 201.4 g (i.e., 135.9 g) would mean an additional daily requirement of 4.85 g fat/day, which

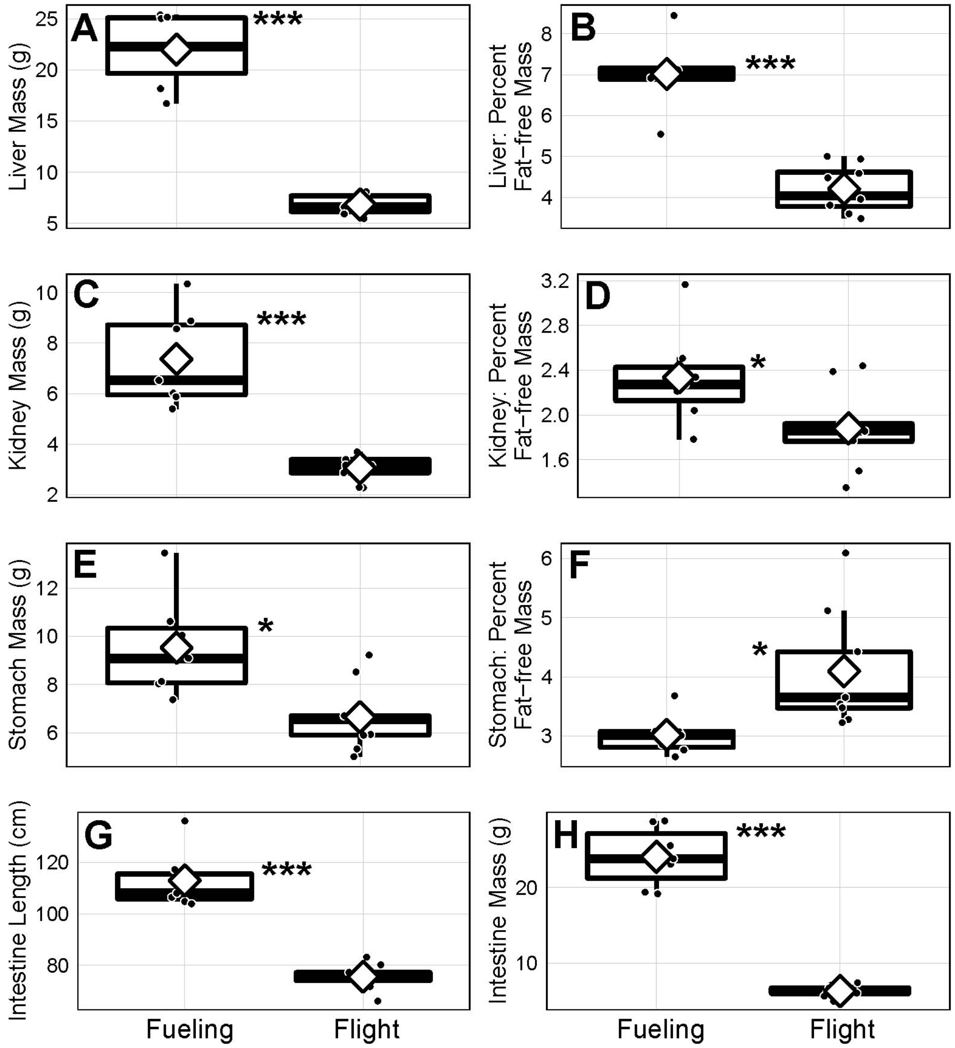

FIGURE 3 | Mass **(A,C,E,H)**, mass relative to fat-free mass **(B,D,F)**, and length **(G)** of body components of juvenile bar-tailed godwits sampled in Alaska, United States, of body components of juvenile bar-tailed godwits sampled in Alaska, United States, during the fueling (*n* = 2 females and 5 males) and flight (*n* = 9 males) annual-cycle stages of their first southward trans-Pacific migration. Horizontal lines represent the median, diamonds the mean, black circles the actual values, boxes the 25th and 75th percentiles, and whiskers the range of values. Statistically significant group differences based on analysis of variance analyses controlling for effects of sex represented by $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

would mean a daily additional energy requirement of 4.85/0.85 (=assimilation efficiency; Kersten and Piersma, 1987) × 40 kJ (=energy density per g fat), or 228 kJ/day. This would add a 58% increase over the maintenance requirement (ca. 400 kJ/day according to Scheiffarth et al., 2002). Thus, during the 28 days of staging following on from the moment of sampling at Egegik Bay, in order to "pay" for a linear increase in fat stores staging bar-tailed godwits would still need to keep eating over 50% more than what would be required to simply maintain energy balance.

This raises a new question regarding the seasonal progression of fat storage and the shrinking of digestive machinery, changes that probably do not happen in linear ways. Specifically, when in the departure process do the various physiomorphic transformations take place and what cues them? For example, if birds reach some set point of fuel and then cue on a synoptic weather event to begin the transition, they likely have 2–5 days over which to at least reduce the digestive machinery. During this period, the flight muscles may also be tuned to the impending endurance challenge. The prolonged circuitous bouts of flight seen at Egegik Bay and Nelson Lagoon which were only observed in the few days before departures (R.E. Gill personal observation), raise the question whether these flights provide the required training to shape the flight machinery. It is also possible that, instead, the flights function in a more social context related to "coordinating" departures of birds in similar condition (Piersma et al., 1990).

More questions remain, as the inferences on the bodily transition were made on the basis of comparisons of juveniles before their first trans-Pacific flight rather than experienced adults. In view of the similar water percentages in the fat-free fraction of the body, it is unlikely that tissue maturation (see Starck and Ricklefs, 1998) contributed to some of the reduction in overall water mass between fueling and flight samples. Nevertheless, this leaves open the option that some of the increase in fat mass between sampling periods could be due to completion of growth and consequent reallocation from growth to energy storage. We now need comparative work on adults to illuminate these questions.

The experimental finding that captive and relatively immobile shorebirds can build flight muscles without exercise (Dietz et al., 1999), nicely predates Hoppeler's (2016) conclusion that "Muscle bulk and composition can be changed without invoking endurance or strength training paradigms, depending on the needs of a species. Available signaling networks can be invoked by any adequate internal or external cue." Thus, whereas for exercise physiologists it may not come as a surprise that bar-tailed godwits can do what they do, the precise time path of physiomorphic preparation for the extreme endurance exercise required for trans-Pacific and other long-distance migrations still needs to be revealed. Positioned in ecological context, with reference to food availability, access to food resources, time-activity budgets, diets, and the danger of predation, they may lead to a better appreciation on the creative evolutionary interface between organisms and environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Data used in this study are publicly available from the U.S. Geological Survey (https://doi.org/10.5066/P9GIQ8J2).

ETHICS STATEMENT

The animal study was reviewed and approved by the Alaska Department of Fish and Game and the USFWS Migratory Bird Permit Office.

AUTHOR CONTRIBUTIONS

TP and RG conceptualized the study. RG collected the data. DR analyzed the data. All authors wrote the manuscript and approved the final manuscript.

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Serendipity played a large role in bringing this story together. Ben Weinrick, Cold Bay Long Range Radar site technician had the foresight to contact Chris Dau, the local U.S. Fish and Wildlife Service (retired) biologist, within minutes of when the godwits collided with the dome. Had Weinrick waited until the morning, scavenging foxes and gulls would have undoubtedly found the birds. Dau, in turn, realized not only the significance of the birds in terms of documenting trans-oceanic migration but also the importance of recording timely meristic data from fresh specimens. And we of course owe special thanks to Leigh H. Fredrickson, University of Missouri-Columbia (Emeritus) for his lab's detailed compositional analyses of the Cold Bay specimens. Anne Dekinga helped us during the collection of the Egegik birds and their laboratory analysis in Netherlands. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Circadian and Seasonal Patterns of Body Temperature in Arctic Migratory and Temperate Non-migratory Geese

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Arctic migration presents unique challenges to circadian physiology. In addition to the metabolic cost of maintaining a relatively high body temperature (Tb) above ambient temperature, migratory birds are also exposed to rapidly changing light conditions as they transition between light-dark cycles and a 24-hour polar day. A previous study suggested that Arctic-migratory barnacle geese (*Branta leucopsis*) may utilise adaptive heterothermy (i.e., a controlled decrease in core Tb) during and around the autumn migratory period in order to minimise the metabolic cost of migration, but the impact of seasonally changing daylight conditions on other parameters of the circadian profile of Tb in these geese remained obscure. Here, we provide a detailed comparative analysis on the circadian rhythm of Tb and its seasonal development in free-living barnacle geese from three study populations that differ in their migratory behaviour and in the environments they occupy. We recorded abdominal Tb in non-migratory geese from a temperate breeding colony in Netherlands and in migratory geese from a colony in the Russian low Arctic, and analysed these data together with previously published Tb data on geese from a migratory colony in the high Arctic of Svalbard. We found that the circadian Tb profile in the barnacle goose was well aligned with the daily and seasonally changing daylight conditions. In the migratory populations, a fast re-entrainment of the rhythm and its phase was observed when *zeitgeber* conditions changed during migratory movements. The circadian rhythmicity of Tb was lost once the geese encountered permanent daylight at their northern staging and breeding sites. Circadian Tb rhythmicity was re-established when the period of permanent daylight ended, at rates corresponding to rates of seasonal changes in daylength in the high and low Arctic. Although our data corroborated findings of a decrease in daily mean Tb before autumn migration in both migratory populations in this study, the pre-migratory decrease in Tb was less drastic than previously reported. Moreover, in contrast to previous study, the decrease in Tb stopped at the onset of migration. Overall, our data reveal no evidence that heterothermy in the barnacle goose is functionally linked to migration.

Keywords: activity, arctic, arrhythmicity, body temperature, circadian rhythms, herbivore, heterothermy, migration

INTRODUCTION

Endotherms, such as birds and mammals, actively regulate their body temperature (Tb), enabling them to perform their activities largely independent of temporal and spatial variation in ambient temperature. As a result, endotherms generally inhabit broader regions and more diverse habitats than ectotherms (Gaston, 2003). In birds, a vivid expression of the independence from environmental constraints that can be gained from thermal independence is long-distance migration over large environmental gradients, such as between Arctic breeding grounds and southern wintering sites. Animal migration has evolved in many species as an adaptation that enables them to exploit spatially distant seasonal peaks of resource abundance and avoid resource depression (Alerstam et al., 2003; Holt and Fryxell, 2011).

When travelling to Arctic breeding grounds, fast-moving animals such as birds experience rapid changes in light conditions along the route, and finally arrive into a 24-h polar day. These rapid changes in light conditions present a unique challenge in the context of circadian physiology (Gwinner and Brandstätter, 2001). Circadian rhythms refer to the coordinated, endogenously driven, 24-h patterns of physiology and behaviour, such as activity and Tb, that improve the adaptability of an organism by allowing it to anticipate predictable changes in the environment (Dunlap et al., 2004). The daily cycle of light and darkness, due to the rotation of the earth around its axis, is one of the most predicable changes in the environment. Accordingly, light is the most salient *zeitgeber*, or entrainer, of circadian rhythms for most surface-dwelling organisms, including birds (Golombek and Rosenstein, 2010; Cassone, 2014). When an organism with a circadian rhythm is exposed to a change in the timing of a *zeitgeber*, like light, there is a temporary mismatch between the internal state of the animal, and the environment in which it is performing. Over time, the internal rhythm adjusts to this disruption, and eventually alignment is restored. However, cognitive performance may be less than optimal during realignment (Tapp and Natelson, 1989). Temporal mismatches between behaviour and environment can lead to a higher risk of being predated (DeCoursey et al., 2000), or alter metabolic homeostasis (Fatima and Rana, 2020). As such, maintaining a degree of plasticity in circadian timekeeping is likely advantageous for Arctic-migrating species (Helm et al., 2017; Åkesson et al., 2021).

Performing migratory journeys requires both time and energy. To fuel their migratory flights, birds deposit and replenish body stores (mainly fat and protein) by foraging intensively during the pre-migratory period and at stopover sites (Bairlein, 2003; Drent et al., 2007; Eichhorn et al., 2012). The rate of fuelling is a key factor for the overall speed of migration, thereby affecting the prospects for an individual's fitness (Alerstam and Lindström, 1990; Gómez et al., 2017). Because the maintenance of a high and relatively stable Tb is energetically costly for endotherms when facing ambient conditions outside their thermoneutral zone, a potential mechanism that may positively influence the net (re)fuelling rate is a less robust defence of Tb against the cold, thus saving the energy that would be expended on heat production (Geiser and Brigham, 2012). Such adaptive heterothermy, which refers to the abandonment of homeothermy in favour of reducing metabolic costs and improving fuel economy, has been suggested for migrating bats and hummingbirds using torpor (Hiebert, 1993; McGuire et al., 2014). But shallower heterothermy was observed also in a songbird (i.e., blackcap *Sylvia atricapilla*) during nocturnal rest at a migratory stopover (Wojciechowski and Pinshow, 2009), and in the barnacle goose *Branta leucopsis* during autumn migration from Svalbard to wintering grounds in Scotland (Butler and Woakes, 2001).

The latter example is intriguing, because Tb decreased during both the resting phase and the active phase of the diel cycle. Together, those decreases caused a progressive decline in mean abdominal temperature of more than 4°C from few days before until approximately 20 days after the start of the autumn migration. Thus, heterothermy persisted throughout and for several days after the end of migration (Butler and Woakes, 2001). The energy savings that result from this heterothermic response may significantly influence the economy of fat stores during and after migration. Such pronounced heterothermy is remarkable for such a large bird especially during a period when exercise is such an important aspect of daily life (McKechnie and Lovegrove, 2002; Eichhorn et al., 2011). For example, in non-migratory greylag geese *Anser anser*, seasonal differences in mean Tb throughout the entire annual cycle (lowest in winter and peaking in summer) were only 1°C (Wascher et al., 2018).

For our analyses in this paper, we took advantage of naturally occurring variations in the migratory ecology of free-living barnacle geese. Specifically, we recorded abdominal Tb in non-migratory geese from a temperate breeding colony in the Netherlands, and in migratory geese from a colony in the Russian low Arctic. To re-examine previous findings of seasonal heterothermy reported for this species (Butler and Woakes, 2001), we also included in our analysis previously published Tb data from migratory geese breeding in the high Arctic of Svalbard (Portugal et al., 2019). Geese from the Russian and Dutch study colonies share common wintering grounds stretching along the mainland coast of the North Sea and southwestern Baltic. However, the geese from the Dutch colony generally stay in the

Netherlands year-round. Barnacle geese from the Russian colony migrate from their wintering grounds via potential stopovers in the Baltic, and further a cross the boreal belt to reach Arctic staging sites in the White Sea (e.g., Dvina river delta and Kanin Peninsula). Approximately 2–3 weeks later, they reach breeding sites further northeast along the coast of the Barents Sea, including our study site at Tobseda (Eichhorn, 2005; Eichhorn et al., 2006). Traditionally, the time between mass departure from the wintering grounds (mid-April) and arrival at Arctic staging sites with continuous daylight conditions (after mid-May) is approximately 1 month, because of prolonged staging in the Baltic. However, since the 1990s, an increasing number of barnacle geese heading for the Russian Arctic have delayed their departure from wintering grounds until well into May. Individuals that follow this new strategy do not arrive later in the Arctic, but shorten, or entirely skip, staging in the Baltic, thereby experiencing changes in daylight conditions more rapidly as they move to the Arctic (Eichhorn et al., 2009; Jonker et al., 2010). By providing a detailed comparative analysis on the circadian and seasonally patterns of Tb in these three populations, we attempt to shed more light on chronobiological aspects in this Arctic migratory bird as well as on the question of whether heterothermy and migration might be functionally linked.

MATERIALS AND METHODS

Study Populations and Data Collection

We studied migratory barnacle geese breeding in the Russian low Arctic and non-migratory geese breeding in the Netherlands. Additionally, we retrieved published data from migratory barnacle geese breeding in high Arctic Svalbard. The latter consist of data on abdominal body temperature (Tb) and heart rate of geese studied in a colony at Ny-Ålesund (78°55′N, 11°56′E) between summer 1999 (implantation of recording devices) and summer 2000 (retrieval). The original Tb and heart rate data were recorded continuously at a sampling rate of 5 s in that study, however, the data we accessed had 15-min averages, available from five individuals (Portugal et al., 2019). Following Portugal et al. (2012), periods of migratory flight were determined from heart rate. For data and further details we refer to Portugal et al. (2019) and references therein. Svalbard barnacle geese spend the winter in the Solway Firth, Scotland (United Kingdom). They leave their winter grounds from mid-April onward for a prolonged staging period along the Norwegian coast (mainly in the Helgeland district) before they travel further to reach Svalbard in the second half of May (Black et al., 2007; Shariati-Najafabadi et al., 2016). The geese leave the Svalbard archipelago again toward the end of September, often after a stopover on Bjørnøya (Bear Island), and fly again along the Norwegian coast but, in contrast to spring, make only brief stops and arrive back in their winter grounds at the end of September or early October (Butler et al., 1998). In Russia, we studied barnacle geese in a colony at Kolokolkova Bay near the abandoned settlement of Tobseda (68°35′N, 52°20′E), situated along the coast of the Barents Sea. Barnacle geese from a non-migratory population were studied in a colony in the Delta area in the southwest of the Netherlands (51°40′N, 4°14′E). More details on these study sites and populations can be found in Van der Jeugd et al. (2003, 2009) and references therein. For a map showing the routes of barnacle geese tracked to their breeding grounds in Svalbard and Russia see Shariati-Najafabadi et al. (2016).

In our Russian and Dutch study sites, geese were captured during their post-breeding wing moult in summer 2018, by driving the flightless geese into a catching pen. We selected adult female geese, which were identified by cloacal examination (sex) and the absence of any (not yet moulted) juvenile feathers. Hence the birds were most likely mature birds in their third calendar year or older (Cramp and Simmons, 1977). Additionally, we selected one immature female goose (CHL0289) that was born and ringed in the Netherlands (NL) in 2017. Selected geese (21 in Russia [RUS], 15 in NL) were instrumented with a solar-powered tracking device (36 × 70 × 15 mm, 24 g) placed on the back by a harness (16 g) recording GPS location and 3D accelerometry (GsmRadioTag, Milsar Technologies S.R.L.), and an implantable data logger (46 × 15 mm, 19 g) recording Tb and heart rate (DST centi-HRT, Star-Oddi). Tb was measured at ±0.2°C accuracy, and all loggers were calibrated by the manufacturer at 41 setpoints within the range of 5 to 45°C. The geese were also banded with individually inscribed darvic leg-rings to allow recognition in the field. Total instrument mass equated to 3% of average body mass. The sterilised data logger was implanted into the abdominal cavity via mid-line incision in skin and body wall while the bird was under isoflurane gas anaesthesia. Using silk suture, the logger was fixed at one end to the cartilage of the xiphoid process, and at the other end to the body wall. Dissolvable suture was used to close the muscle layers and skin. After surgery, the birds were monitored for at least 4 h while they were kept in an outdoor pen, before they were released back to the wild together with others in a small flock.

The data loggers were programmed to record Tb continuously every 10 min, except during the following periods when the interval was reduced to 5 min: from 20 September to 22 October 2018 (both NL and RUS geese) and again from 7 April to 23 June 2019 (NL geese) or 27 April to 13 July 2019 (RUS geese). Attempts to recapture the geese for retrieval of loggers were made in the Russian study site during wing-moult in summer 2019, and in the Dutch study site during summer 2019 and 2020. Unfortunately, most of the GPS trackers stopped working at an early phase of the study, which hindered the detection and recapture of study subjects, particularly at the Russian site. Recaptured focal geese were euthanised with an overdose of pentobarbital (Euthesate) injected intravenously, and all instruments were removed. Some data loggers were also sent in by hunters, who shot geese during their regular hunting activities, thereby retrieving loggers from one RUS goose and three NL geese. In total we gathered Tb data of various recording length from three RUS geese and 11 NL geese, of which three (RUS) and eight (NL) recordings were included in the present analysis (other NL geese had too short recordings as they were shot by hunters within few weeks of implantation).

Daily ambient mean, minimum and maximum temperature measured at meteorological stations in Ny Ålesund (breeding site of geese studied in Svalbard), Eskdalemuir (20 km from the

Caerlaverock nature reserve in Scotland, the principal wintering site for Svalbard barnacle geese), Naryan-Mar (103 km from the Russian study colony), and Wilhelminadorp (33 km from the Dutch study colony) were retrieved from the European Climate Assessment and Dataset at http://www.ecad.eu (Klein Tank et al., 2002). For the computation of sunlight phases we used the R-package "suncalc" (Thieurmel and Elmarhraoui, 2019). Civil twilight refers to sun angles between 0° (i.e., sunset or sunrise) and −6°, and nautical twilight to sun angles between −6° and −12°.

Data Analysis

Periodicity in the Tb data was determined using Lomb-Scargle periodogram analysis as implemented in the "lomb" package in RStudio (R version 4.0.4 for Windows) (Ruf, 1999; RStudio Team., 2020; R Core Team., 2021). Five-day blocks of data were scanned at an oversampling rate of 200 to detect significant ($p < 0.001$) periodicity between 6 to 30 h in duration. Because the normalised power (PN) is sensitive to sampling rate of the Tb time series, data were re-sampled at 30 min interval to ensure consistency where comparisons were made between study populations that were sampled at a different time interval. Otherwise, the native sampling intervals were used. Periodogram analyses confirmed that, when rhythmic, the Tb data typically contained significant periods at 24 h and occasionally at 12 h (**Supplementary Figure 1**). Accordingly, we used the "cosinor" (Sachs, 2014) and "card" (Shah, 2020) packages in RStudio (R version 4.0.4 for Windows) to fit multicomponent cosinor models to the daily Tb data, comprising of a 24 h period and a 12 h harmonic. The multicomponent model is described by the following formula:

$$Y = M + A1 \times cos(2\pi(t)/24 + \varphi 1) + A2 \times cos(2\pi(t)/12 + \varphi 2)$$

Here, M refers to the midline estimation statistic of rhythm (MESOR), $A1$ and $A2$ are variables defining amplitude, $\varphi 1$ and $\varphi 2$ are variables describing phase, and t is time in hours. To describe the circadian profile, the MESOR, peak, trough, and global amplitude, defined as half of the distance between the peak and the trough of an oscillation, were extracted from the model. Since the data were sampled at equidistant intervals and an integer number of cycles were analysed, the MESOR is equal to the arithmetic mean (Refinetti et al., 2007). In addition to the PN, we also used the coefficient of determination (R^2) multiplied by 100 as a measure of the relative robustness of the circadian rhythm, herein referred to as percent rhythm (Refinetti et al., 2007).

We used the daily acrophase, defined as the timing of the daily maximum, to infer the timing of the Tb rhythm and thereby examine entrainment of the circadian rhythm. To focus on the timing of the circadian (i.e., 24 h) rhythm and avoid the confounding effects of the 12 h harmonic, we extracted the acrophase from the following single component cosinor, instead of the multicomponent cosinor model:

$$Y = M + A \times cos(2\pi(t)/24 + \varphi)$$

As above, M refers to the MESOR, A is a variable defining the amplitude, φ is a variable describing phase, and t is time in hours. **Supplementary Figure 2** shows examples of the multicomponent and single-component models. Linear mixed effects analysis of the relationship between acrophase and stage of migration were performed using R and the "lme4" package (Bates et al., 2015) with stage of migration entered as a fixed effect, and individual ID as random effect. Visual inspection of residual plots revealed no heteroscedasticity or deviations from normality. The focus of our present analyses was on the circadian and seasonal Tb pattern related to different migration strategies and breeding environments. We deliberately excluded the breeding period itself to avoid additional interference from incubation rhythms.

Two-way mixed ANOVA (as implemented in IBM SPSS, Version 25.0) was used to evaluate differences in the daily mean Tb among the three goose populations (between-subjects factor) across three points in time/state around autumn migration (within-subjects factor), which allowed also to test for an interaction, i.e., to evaluate if any changes in Tb over time differed among populations. Data were log-transformed before analysis to improve distributional properties (normality, variance). Normality was assumed based on Shapiro-Wilk tests (all tests yielded $P > 0.05$). Homogeneity of variances was assumed based on Levene's test using the median, because of skews or uncertainty in distribution (all tests yielded $P > 0.05$). Homogeneity of covariances was assumed based on Box's M-test (yielding $P > 0.001$); a lower P-value is justified because of the high sensitivity of this test (Tabachnick et al., 2019). Sphericity was assumed based on Mauchly's test of sphericity (yielding $P > 0.05$). Sample size in the data set was low, but multivariate analyses of repeated measures ANOVAs seem robust even when performed with sample sizes as low as three as long as data are normally distributed (Oberfeld and Franke, 2013). Based on a significant interaction in the mixed ANOVA, we followed up by running tests for single (main) effects (i.e., one-way models testing the effect of one factor at each level of the other factor). If a single main effect was significant, multiple pairwise comparisons were run to determine which groups were different. A Sidak correction was applied in multiple (pairwise) comparisons. Effects are reported as significant at $P < 0.05$.

RESULTS

Circadian Rhythmicity of Tb

Variation of Tb in the barnacle goose was well aligned to the daily and seasonal daylight conditions (i.e., light-dark cycle of the day) in areas and seasons with a day-night cycle (**Figure 1**, **Supplementary Figure 3**). Tb rose sharply around dawn and declined sharply around dusk, resulting in a robust and prominent circadian (24 h) rhythmicity with a mean acrophase of 13.6 ± 2.2 h (mean ± SD) when geese were in their wintering grounds (between November and March). Strong and rapid changes in the acrophase occurred during migratory movements and when the birds experienced continuous daylight conditions (**Figure 2**). The Tb rhythm and its acrophase was quickly

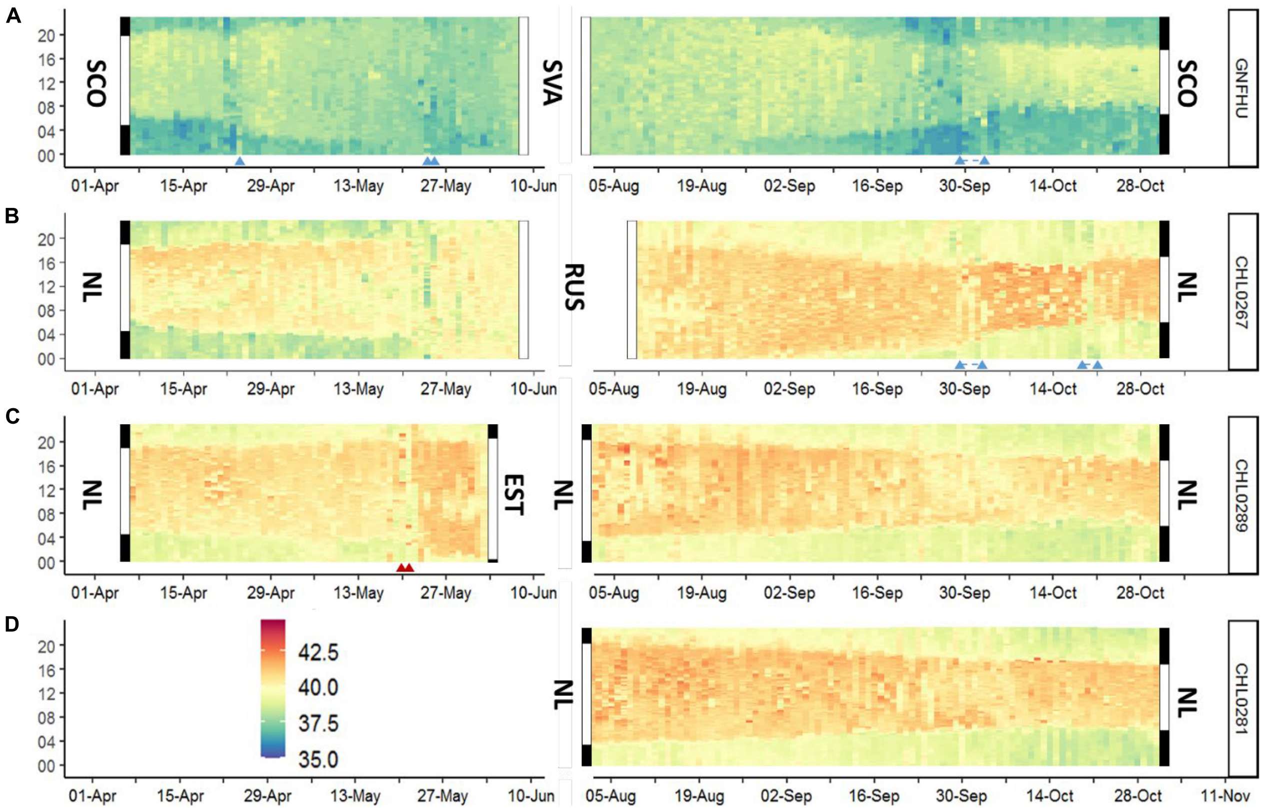

FIGURE 1 | Examples of diel (vertical axis, in hours) and seasonal body temperature (Tb, in °C) patterns in individual barnacle geese (ID given on the right of each plot) from the Arctic-migratory populations **(A**: Svalbard, **B:** Russia) and the non-migratory population **(D**: Netherlands). Panel **C** refers to an immature individual (CHL0289; born in NL in 2017) that flew in spring 2019 to Estonia. Vertical bars at the start and end of the Tb plots indicate the period of daylight, including civil twilight (white) and night (black) in the wintering and breeding areas (SCO, Scotland; SVA, Svalbard; NL, Netherlands; RUS, Russia; EST, Estonia). Time of day refers to GMT in all panels. Migratory movements are indicated by triangles; if the triangles are connected by dashed lines, then migratory activity was recorded throughout this period. See also **Supplementary Figure 3** for further examples.

adjusted to phase shifts in diel light conditions resulting from longitudinal movements, which was most apparent during the autumn migration when the birds experienced diel light-dark cycles throughout (**Figures 1**, **2**; **Supplementary Figure 3**).

The rhythmicity of Tb was lost in the migratory geese once they encountered continuous daylight conditions at their northern staging and breeding sites (**Figure 3**). After the period of continuous daylight had ended, rhythmicity resumed (**Supplementary Figure 1**), however, at a different pace in the high and low Arctic (**Figure 4**). In Svalbard, continuous daylight lasts longer throughout the season and, accordingly, the geese remained arrhythmic for longer. Virtually with the first day of sunset and the return of civil twilight on 28 August, circadian rhythmicity resumed. In contrast, at the Russian site, the first sunset was on 21 July, but circadian rhythmicity of Tb was resumed only after approximately 20 August, when the geese experienced already several hours of civil and nautical twilight. Geese in Netherlands generally showed stronger Tb rhythmicity than geese in the Arctic summer, although rhythmicity increased also in the Dutch geese toward the end of the temperate summer.

Seasonal Heterothermy

A visual inspection of the individual traces shown in **Figure 5** reveals that geese from all three populations exhibited a gradual decline in the daily mean of Tb throughout early autumn. In the non-migratory geese, the decline came to a halt in the 2nd week of October. In the migratory geese, the decline stopped with the onset of migration. Most geese departed when ambient temperatures fell to or below 0°C (i.e., when frost first occurred). Tb increased again during migration, albeit to a lower level than had been observed in summer. The increase happened most rapidly in Svalbard geese, which completed the migratory journey within a short period. Once the geese arrived at their wintering grounds or pre-wintering staging sites, the Tb remained relatively stable.

Any between-individual comparison of absolute Tb values is hampered by the nature of individual measurement variation that cannot be accounted for (e.g., differences in the exact position of the temperature probe). However, within-individual variation throughout autumn appeared higher in geese from Svalbard than in geese from Russia or Netherlands (**Figure 5**). To summarise and formally test for Tb patterns observed around

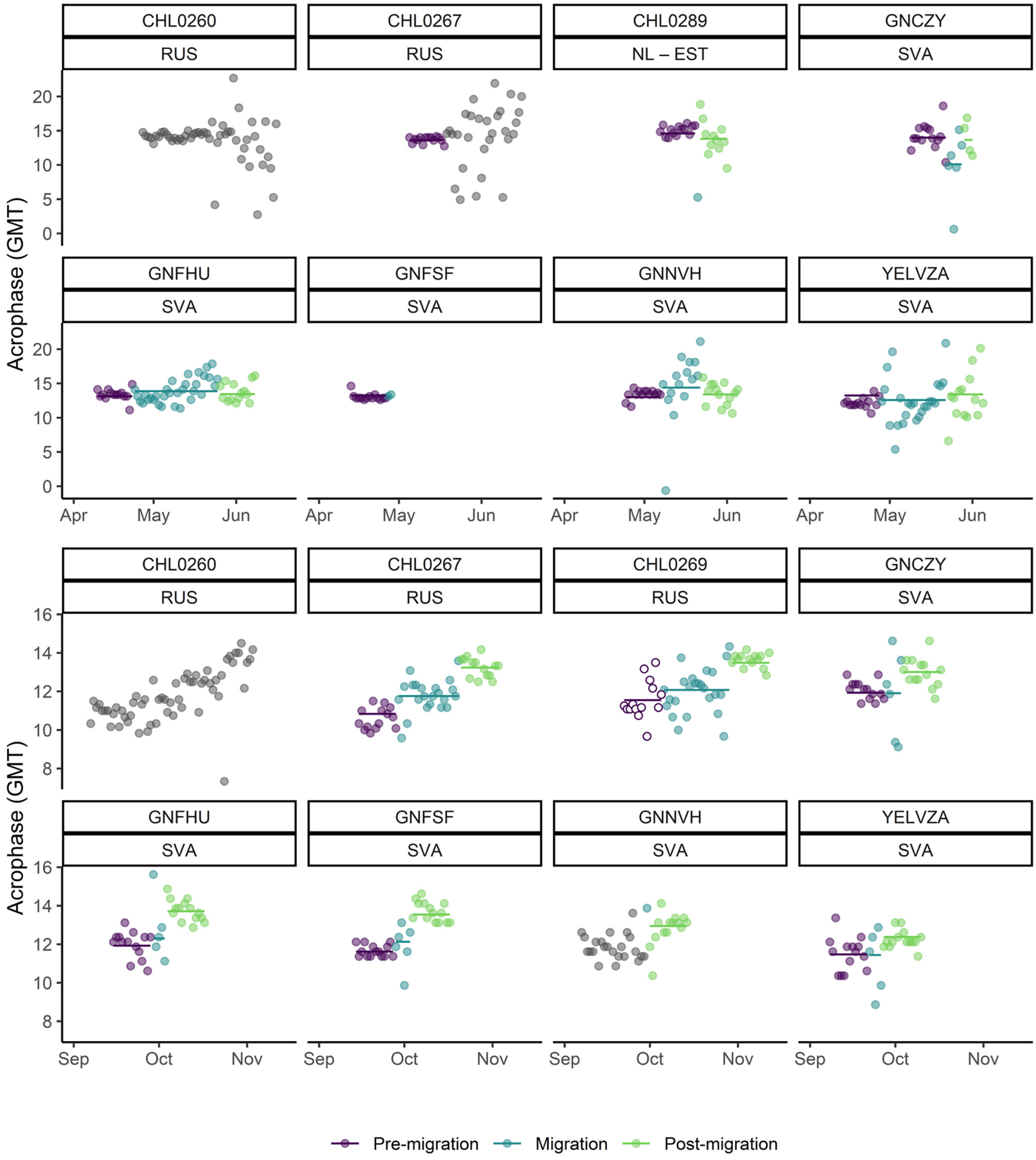

FIGURE 2 | Daily acrophase of individual Tb rhythms over spring and autumn in barnacle geese from the Svalbard (SVA) and Russian (RUS) populations (individual ID is given on top of each plot). Included is also goose CHL0289 from the Netherlands population (NL) that migrated to Estonia (EST) in the Baltic in spring. The migration period was determined from heart rate (SVA) or GPS locations (RUS, NL), and the 14 days immediately preceding and following migration are plotted as the pre-and post-migratory periods. Data from days where the migratory stage of the bird could not be inferred from either heart rate or GPS are plotted in grey. Open circles in the panel for CHL0269 refer to a period when the bird was located at a staging site (Kanin Peninsula) southwest of the breeding site. Model fits from linear mixed effects analysis of acrophase during each stage of migration are shown as horizontal lines.

autumn migration, we selected the day with the lowest daily mean Tb recorded during 21 September to 21 October (thereby effectively aligning the individual time series to a common "onset of migration" in migratory geese) and compared that time to a premigration summer mean Tb recorded during 10 to 20 August (when both migratory populations were still arrhythmic) and to a postmigration mean Tb recorded during 23 October to 15 November. The intra-individual decline in the daily mean Tb from summer to autumn amounted to, on average, 1.4°C in geese from Svalbard, and 1.0°C in geese from Russia and Netherlands (**Table 1**, **Supplementary Figure 4**). While there was an overall significant change in Tb across the three points in time / state (two-way mixed ANOVA $F_{2,26} = 200.60$, $P < 0.001$), this change differed among populations (time × population interaction, $F_{4,26} = 6.83$, $P = 0.001$). Contrasts revealed that the interaction was significant when "autumn low Tb" and "summer mean Tb" ($F_{2,13} = 10.41$, $P = 0.002$) were compared, but not when "autumn low Tb" and "postmigration mean Tb" ($F_{2,13} = 3.61$, $P = 0.057$)

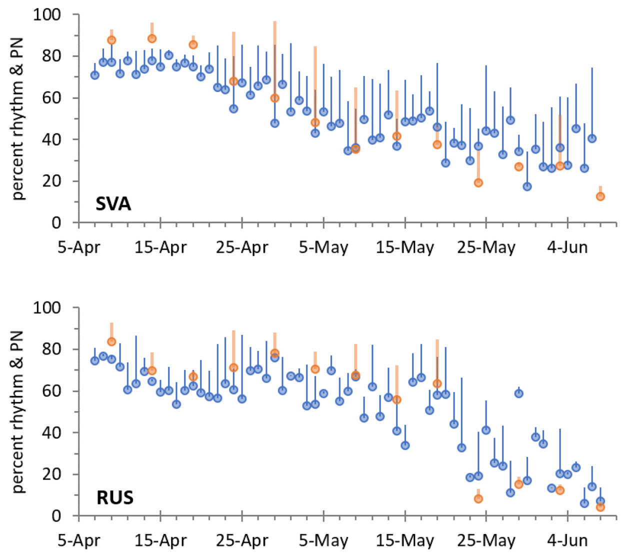

FIGURE 3 | Course of circadian rhythmicity of Tb in barnacle geese during spring, encompassing the time before, during, and after migration to their Arctic breeding grounds in Svalbard (SVA) and Russia (RUS). Plotted are percent rhythm (mean + SD over individuals, blue symbols) calculated from daily cosinor analysis, and normalized power (PN, mean + SD over individuals, orange symbols) at a period length of 24 h calculated from Lomb-Scargle periodogram analyses on 5-day intervals (both left axis).

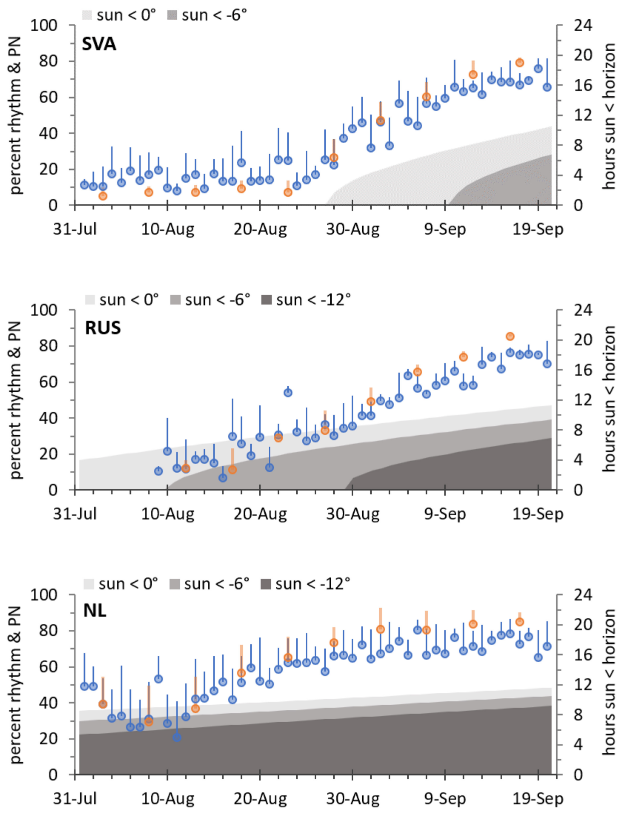

FIGURE 4 | Course of local photoperiod and circadian rhythmicity of Tb in barnacle geese at the end of summer while staying in Svalbard (SVA), in Russia (RUS), or in the Netherlands (NL). Plotted are percent rhythm (mean + SD over individuals, blue symbols) calculated from daily cosinor analysis, and normalized power (PN, mean + SD over individuals, orange symbols) at a period length of 24 h calculated from Lomb-Scargle periodogram analyses on 5-day intervals (both left axis). The amount of time the sun was at certain angles below horizon (right axis) is shown by shades of grey representing categories of, respectively, civil twilight, nautical twilight, and darker.

were compared. In other words, geese in Svalbard exhibited a stronger autumn decline in Tb than did geese in Russia or Netherlands (**Supplementary Figure 4**). The overall main effect of population was significant ($F_{2,13}$ = 5.20, P = 0.022) due to generally lower Tb values in Svalbard geese than in geese from our study. One-way models and *post-hoc* tests indicated that, for each population, Tb differed in all comparisons among the three points in time / state ($P \leq 0.014$) except for the comparison of mean Tb in summer and after migration in Russian geese (P = 0.064). The daily mean Tb in the Russian geese did not differ from that in Netherlands geese at any of the three points in time / state ($P \geq 0.968$).

The decline in mean (MESOR) Tb was attributable mainly to a deeper trough of Tb during the resting phase of the diel rhythm, and much less to a change in the peak Tb, resulting in an increase in the amplitude of the rhythm (**Supplementary Figures 5**, **6**). However, in the Arctic, the peak Tb also showed a slight decline leading up to the onset of migration, followed by a more noticeable rebound thereafter.

DISCUSSION

We investigated the Tb patterns of barnacle geese from three study populations, which varied in their migratory strategy and also in the environments they occupied, most notably in solar light regime. Typical for a (largely) diurnally active species, Tb levels were higher during the light phase, and lower during the dark phase, of the diel period. The geese showed a robust circadian Tb rhythm, reflected by increased percent rhythm and normalised power corresponding to a 24-h periodogram, whenever a dark-light cycle was present. It is likely that daylight functions as the dominant *zeitgeber* that entrains their endogenous circadian clock. A fast re-entrainment of the Tb rhythm, shown by shifts in acrophase, which indicates the timing of the rhythm, was observed when *zeitgeber* conditions changed during migratory movements. Circadian rhythmicity disappeared under permanent light of the polar day, while the geese maintained a relatively high Tb during that time. There was a modest decrease in the daily mean Tb throughout autumn, which was more pronounced in Svalbard geese than in geese from Russia or Netherlands. However, the observed Tb changes during the pre-migratory period were of small magnitude compared to a previous study that was conducted on barnacle geese from the Svalbard colony. Moreover, and in contrast to the previous study, Tb increased again as soon as the migratory geese left their Arctic (post-) breeding grounds and returned to their wintering areas. Below we discuss the implications of our findings in more detail.

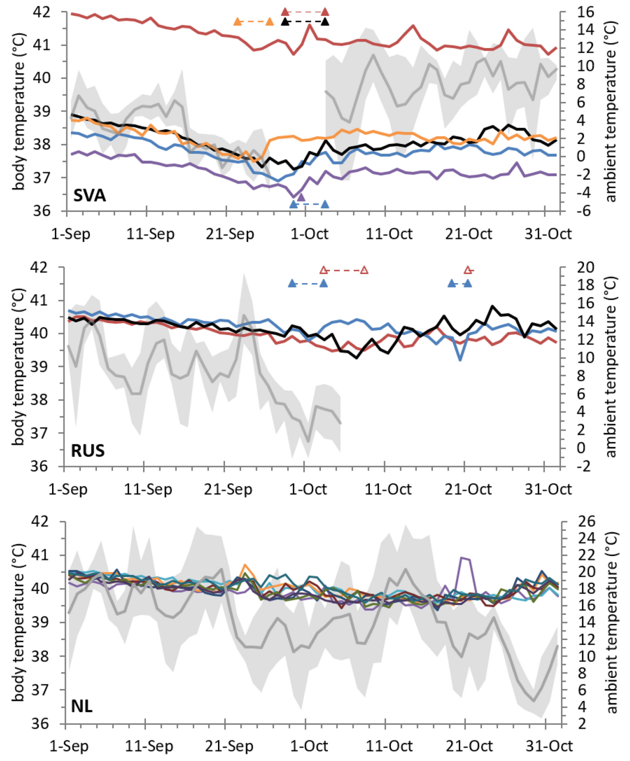

FIGURE 5 | Daily Tb (left axis) of barnacle geese before, during, and after their autumn migration from Svalbard (SVA) to the United Kingdom, from Russia (RUS) to (pre-) wintering grounds in the SW Baltic and Wadden Sea, and of non-migratory individuals in the Netherlands (NL). Shown is the MESOR Tb as modelled from a multi-component cosinor analysis, which equated to daily mean Tb in these data. Migratory movements are indicated by triangles matching in colour with the individual Tb series; if the triangles are connected by dashed lines, then migratory activity was recorded throughout this period. The timing of movements was derived from individual heart rate (SVA) or GPS tracking (RUS); in one case (open triangles) it was estimated from obvious phase shifts observed in Tb data (**Figure 1**). The grey line and the borders of the grey shaded area surrounding it refer to daily mean, maximum, and minimum ambient temperature (right axis) as measured in the breeding area before most individuals departed; for Svalbard geese (with a condensed migratory period) ambient temperature is also shown in their Scottish wintering area.

Circadian Rhythmicity of Tb

The seasonal change in day length (photoperiod) increases with latitude. It has been hypothesized that, for diurnal animals, the available daylight can be a limiting resource that may have favoured the evolution of latitudinal migration. Latitudinal migrants can benefit from a relatively longer photoperiod at higher latitudes, allowing them to prolong their daily activity and foraging time (Sockman and Hurlbert, 2020). In line with this hypothesis are observations of a strong positive relationship between daylight availability and activity in various species of latitudinal migrants (Pokrovsky et al., 2021), however, in those studies, no distinction was made between foraging and non-foraging activities. In the present study we can infer activity patterns of barnacle geese from their Tb patterns (see **Supplementary Figure 7** for an illustration of this relationship). The geese prolonged their daily activity in response to longer daylength until they became active around the clock under continuous daylight. That prolonged activity in this species is mainly devoted to foraging is supported by the finding that the daily gain in body mass increased with the latitude of the staging site (and thus daylength) during the spring migration (Eichhorn, 2008). Intensive foraging continues upon arrival at Arctic (pre-)breeding grounds where the stores needed for clutch production and incubation are replenished (Hübner, 2006; Eichhorn et al., 2010).

After breeding, the migratory geese need to build up body stores for the autumn migration. It is perhaps for this reason that the Russian geese in the low Arctic remained largely arrhythmic, despite experiencing again light-dark cycles, during the first half of August (**Figure 4**). Geese are known to undertake additional nocturnal foraging during low light conditions, such as during twilight and moonlit nights, if they need extra intake to balance their energy budget (Lameris et al., 2021). Presumably, the Russian and Svalbard geese have similar energetic requirements when they are preparing for autumn migration (of a similar distance). However, at times when the geese in Svalbard can still benefit from the longer polar days, the geese in low Arctic Russia may need to extend their foraging activity into the dark phase of the day. The adaptive value for the geese of staying active around the clock, even in the presence of the usual external *zeitgeber*, is presumably to balance energy requirements in the context of migration. In contrast, as soon as the high Arctic Svalbard geese experienced the first days with sunset in late August, they aligned their primary resting phase to the dark phase of the day, which resulted in robust rhythmicity (**Figure 4**, **Supplementary Figure 1**). These different responses could illustrate the plasticity in individual timekeeping and / or in the behavioural and physiological output of timekeeping systems, and emphasize the functioning of the circadian system in an ecological context (Helm et al., 2017).

A malleable circadian pacemaking system, which can be quickly adjusted to changing *zeitgeber* conditions, is of advantage also during migratory movements. The free-ranging geese studied here showed a rapid re-entrainment of rhythm and its acrophase. Åkesson et al. (2021) demonstrated a fast re-entrainment of circadian rhythms in two songbird species by experimentally changing the photoperiod and simultaneously measuring the pattern of migratory restlessness and feeding.

It is thought that the hormone melatonin plays a key role for the malleability of the circadian pacemaker. A degree of malleability is important for maintaining entrainment when *zeitgeber* conditions change quickly, as they do during migration or when the diel amplitude of a *zeitgeber* is low, as it is at high latitudes. Accordingly, a reduced, but still rhythmic, amplitude of melatonin in the plasma has been reported during the polar day and during migration in birds (Gwinner and Brandstätter, 2001; Silverin et al., 2009). The observation of increased melatonin during a migratory stopover implies an entrainable clock during migration, and the stabilisation of circadian rhythms to a new photoperiod during staging periods (Fusani and Gwinner, 2004). Fast re-entrainment of the circadian clock has also been reported

TABLE 1 | Daily mean body temperature (Tb, in °C) in individual barnacle geese from Arctic-migratory populations in Svalbard (SVA) and Russia (RUS), and from a non-migratory population in Netherlands (NL) at three time periods / states around autumn migration.

Population	ID	Summer mean Tb	Autumn low Tb	Postmigration mean Tb	Δ summer to autumn low	Δ autumn low to postmigration
SVA	GNCZY	41.81	40.73	40.81	−1.08	0.08
SVA	GNFHU	38.27	36.91	37.68	−1.36	0.77
SVA	GNFSF	38.82	37.25	38.13	−1.57	0.88
SVA	GNNVH	37.93	36.42	37.05	−1.51	0.63
SVA	YELVZA	38.84	37.49	38.08	−1.35	0.59
SVA	Mean ± SD	39.13 ± 1.54	37.76 ± 1.7	38.35 ± 1.44	−1.37 ± 0.18	0.59 ± 0.3
RUS	CHL0260	40.22	39.48	39.78	−0.74	0.30
RUS	CHL0267	40.40	39.20	40.12	−1.20	0.92
RUS	CHL0269	40.21	39.26	40.16	−0.95	0.90
RUS	Mean ± SD	40.28 ± 0.11	39.31 ± 0.15	40.02 ± 0.21	−0.96 ± 0.23	0.71 ± 0.35
NL	CHL0280	40.19	39.39	39.58	−0.80	0.19
NL	CHL0281	40.68	39.59	39.73	−1.09	0.14
NL	CHL0282	40.50	39.51	39.79	−0.99	0.28
NL	CHL0283	40.44	39.36	40.04	−1.08	0.68
NL	CHL0285	40.55	39.42	39.68	−1.13	0.26
NL	CHL0286	40.42	39.46	39.73	−0.96	0.27
NL	CHL0289	40.48	39.45	39.83	−1.03	0.38
NL	CHL0293	40.43	39.60	39.90	−0.83	0.30
NL	Mean ± SD	40.46 ± 0.14	39.47 ± 0.09	39.79 ± 0.14	−0.99 ± 0.12	0.31 ± 0.16

*Given are mean Tb during 10 to 20 August ("Summer mean Tb"), lowest daily mean Tb found during 21 September to 21 October ("Autumn low Tb") and mean Tb during 23 October to 15 November ("Postmigration mean Tb") as well as the change in Tb from summer mean to autumn low and from there to postmigration mean Tb. The population averages are plotted in **Supplementary Figure 4**.*

for an Arctic resident, the arctic ground squirrel *Urocitellus parryii*, which entrained to new light cycles over 1–2 days (Williams et al., 2017) as opposed to up to a week in laboratory mice (Jagannath et al., 2013). However, as opposed to melatonin in birds, the flexibility of the clocks in mammals is likely linked to vasopressin in the SCN (Williams et al., 2017).

A lack, or at least a strong attenuation, of circadian rhythmicity during the polar day is a common phenomenon among Arctic residents, particularly among non-burrowing herbivores such as reindeer *Rangifer tarandus* (Van Oort et al., 2007; Arnold et al., 2018), muskox *Ovibos moschatus* (Van Beest et al., 2020) and Svalbard ptarmigan *Lagopus muta hyperborea* (Appenroth et al., 2021), although they all show ultradian rhythmicity reflecting cycles of foraging and digestion. Irrespective of the exact mechanisms underlying the seasonal absence of overt circadian rhythmicity (see Bloch et al., 2013 for a discussion of putative mechanisms) the functional significance of around the clock activity in these Arctic herbivores seems mainly related to an increase in foraging activity, which facilitates balancing the energetic costs of reproduction and fattening during the short summer. We expect the same holds for migratory geese that visit the Arctic during summer and benefit not only from abundant food resources, but also the possibility of exploiting them throughout a 24-h foraging day. The combination allows for higher growth rates in Arctic barnacle geese compared to their temperate conspecifics; but, at the same time, the Arctic migrants are forced into running their annual cycle at a higher pace of life (Eichhorn et al., 2019). Additional motivations for geese, besides foraging, to stay active around the clock during the polar day likely include predator evasion and competition for nesting sites. In other species, such as Arctic breeding shorebirds, the competition for mates is another strong motivation, which can lead to "reproductive sleeplessness" during the pre-incubation period (Lesku et al., 2012; Steiger et al., 2013). In geese, however, pair formation takes place outside the breeding grounds, and pairs stay together usually throughout life.

Not all Arctic residents or visitors abolish diel rhythmicity during summer (see Williams et al., 2015 for a review). For certain species and circumstances, it is adaptive to maintain a circadian rhythm. It helps arctic ground squirrels, for example, to reduce the energy required for thermoregulation by aligning their above-ground activity rhythm to the generally warmest period of the day, while they use their burrow as a thermal refuge during the colder period (Williams et al., 2012). During the polar day, diel cycles of light intensity and ambient temperature can still be substantial, leading to daily cycles in the availability of arthropod prey, which may be the proximate reason for persistent circadian rhythmicity in insectivorous songbirds and shorebirds (Steiger et al., 2013). Environmental cues such as light intensity or colour, ambient temperature, or food availability, could act as alternative (low amplitude) *zeitgebers* in the absence of light-dark cycles (Stephan, 2002; Ashley et al., 2013). However, it remains unclear if these rhythms are indeed the output of an endogenous clock or are mediated by masking agents, i.e., a direct response to environmental changes (Ashley et al., 2013; Williams et al., 2015).

As outlined above, activity around the clock under continuous daylight has its obvious benefits, although, one wonders about potential negative consequences of prolonged sleep restriction or fragmentation. A recent study has shown that even low light level sources, like full moon or city sky glow, may be enough to suppress sleep in barnacle geese (Van Hasselt et al., 2021). Sleep restriction is known to compromise performance, health and survival. Indeed, "migratory sleeplessness" in white-crowned sparrows *Zonotrichia leucophrys gambelii* was associated with impaired execution of certain cognitive tasks (Jones et al., 2010). In contrast, "reproductive sleeplessness" shown by pectoral sandpipers during the Arctic breeding season appeared not to impair their performance or survival; in fact, males that slept the least sired the most offspring (Lesku et al., 2012). Under continuous daylight, melatonin secretion is reduced, which is expected to suppress immune function (Weil et al., 2015), and sleep deprivation may further weaken the immune system (Irwin and Opp, 2017). But in a pathogen poor environment like the Arctic, even a compromised immune defence might be sufficient (Piersma, 1997). Finally, sleep restriction in the Arctic geese and other birds for that matter may be less severe as it seems from the absence of diel rhythms. They still take resting bouts, which are also needed for food processing, and their ability for unihemispheric sleep enables birds to maintain vigilance against potential threats in their surroundings while still gaining some sleep (Rattenborg et al., 2000).

Seasonal Heterothermy

Like in our present study, Butler and Woakes (2001) aligned their data to the day when minimum abdominal Tb was reached in autumn by each of the ten Svalbard barnacle geese that they studied, and recorded, on average, a daily (24 h) mean value of 35.9°C, which is 4.8°C lower than the mean of 40.7°C recorded at the end of August. In another three non-migratory (captive-bred) geese held in captivity in Birmingham, they recorded a Tb reduction during autumn of only 1.8°C. These differences led the authors to suggest that the expression of heterothermy and migration (fuelling) might be functionally linked. In present study, however, we found that the average Tb reduction was only 1.0°C, and that reduction did not differ between Arctic-migratory geese from Russia and non-migratory geese from a Dutch colony (**Table 1**, **Figure 5**, and **Supplementary Figure 4**). Seasonal differences in mean Tb of 1°C were reported also for non-migratory greylag geese (Wascher et al., 2018). Although we see somewhat larger Tb reductions of 1.4°C (on average, and up to 1.6°C in one individual) in another study on Svalbard barnacle geese, the pronounced heterothermy as reported by Butler and Woakes (2001) does not seem to represent the norm for barnacle geese from Svalbard or other (migratory) populations.

Even though all data sets discussed here concern measurements of Tb inside the abdomen, measurements at multiple body locations in another bird, the king penguin *Aptenodytes patagonicus,* demonstrate that substantial Tb gradients can occur even within the abdominal cavity, and that Tb fluctuations are expected to increase with distance from the deep body core in the thorax with its constantly active organs, such as the heart and liver (Eichhorn et al., 2011). We positioned temperature probes in the Russian and Dutch barnacle geese in the upper abdomen, approximately three centimetres away from the liver. This may explain the generally higher Tb values in our data as compared to data from Svalbard geese in Portugal et al. (2019), where the Tb probe possibly was positioned further down in the abdomen. However, the position of a temperature probe cannot explain the notable differences between the two studies on barnacle geese from Svalbard. The data from Butler and Woakes (2001) show generally higher Tb values before the strong autumnal decline than all other data sets. Possibly extreme annual differences in weather (including e.g., snow or ice cover that influence access to food) and / or body condition may have played a role in the different levels of heterothermy exhibited by geese from Svalbard in the different studies. Most migratory geese that we examined in the present study left their breeding grounds when ambient temperatures fell to approximately 0°C (**Figure 5**), which is in line with the hypothesis that the onset of frost is a major trigger for the timing of the migratory departure in autumn in geese (Xu and Si, 2019).

The Butler and Woakes (2001) data are exceptional also in other ways. They showed large Tb reductions during both the resting phase and the active phase of the diel cycle. In contrast, our analyses show that the decline in the daily (24 h) mean Tb was far more the result of a decline during the resting phase, whereas the peak Tb during the active phase remained rather high and unchanged (**Supplementary Figure 5**). Perhaps the most remarkable finding in that earlier study on Svalbard geese was that the Tb decline continued throughout, and until several days after, migration, which was evidently not the case in the geese that we examined, in neither Russia or Svalbard. Migration was clearly associated with a rebound in Tb, which was particularly evident when we compared individuals that departed on different dates (**Figure 5**, **Supplementary Figure 5**). A continuous decline in Tb throughout migratory movements seems difficult to reconcile with an expected increase in heat production due to intensive exercise. We also assume that it would be advantageous for geese to forage intensively during daylight hours when they are replenishing body stores during staging and after migration. Moreover, although a lower Tb might save energy, it can impair the ability to fly and increase vulnerability to predation (Carr and Lima, 2013). Therefore, it seems adaptive to restrict more drastic Tb reductions to the resting phase and when geese are safe from predators. The dark period of the day offers geese protection from diurnal predators (e.g., white-tailed eagle *Haliaeetus albicilla*), and by retreating to open water or islands, the geese find additional safety from (nocturnal) mammalian predators (e.g., arctic fox *Vulpes lagopus*, red fox *V. vulpes*).

Autumn is characterized by both a shortening of the daylight period and a decrease in ambient temperature. Studies that have investigated the effect of ambient temperature on heterothermy have found only rather small effects [reviewed in Hohtola (2012)]. Although it is difficult to assess the potential effect of ambient temperature in present study, the course of ambient temperature fluctuated substantially and was often not in line with the gradual decline in Tb (**Figure 5**). A gradually lower rest-phase Tb contributed to an increase in the amplitude of the diel rhythm (**Supplementary Figures 5**, **6**) and to a reduction in the daily mean Tb during autumn. In addition, the nocturnal resting phase became progressively longer during autumn, which also contributed to a reduction in the (24 h) mean Tb. The change in photoperiod, and hence length of the nocturnal resting phase, progresses faster at higher latitudes. This might also be the main driver for the relatively steeper decline in mean Tb in geese from Svalbard as compared to geese in Russia or Netherlands (**Table 1**, **Figure 5**, and **Supplementary Figure 4**). In other words, the daily rate of Tb decline through autumn might be slower at lower latitude just because the daily rate of change in photoperiod is slower. Indeed, once these two variables were evaluated in direct relationship, the population differences in the rate of decline disappeared (**Supplementary Figure 8**). Altogether, our examinations revealed no evidence that heterothermy in the barnacle goose is particularly linked to migration. Future studies are encouraged to investigate how common, and under what circumstances, the remarkable Tb reductions reported by Butler and Woakes (2001) may occur.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal research protocol was approved by the Animal Welfare Committees of the Royal Netherlands Academy of Arts and Sciences (license AVD8010020173788) and the St. Petersburg State University (decision nr. 131-03-2 from 3 April 2018).

AUTHOR CONTRIBUTIONS

GE conceived the study and led the writing of the manuscript. GE, MB, and HJ collected the data. GG and GE analysed the data, and discussed the interpretation and presentation of the results together with SM and MB. AM performed the surgical implantations. MW provided crucial equipment. All authors contributed to drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.699917/full#supplementary-material

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Seasonal Patterns of Fat Deposits in Relation to Migratory Strategy in Facultative Migrants

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Physiological preparations for migration generally reflect migratory strategy. Migrant birds fuel long-distance flight primarily with lipids, but carrying excess fuel is costly; thus, the amount of fat deposited prior to departure often reflects the anticipated flight duration or distance between refueling bouts. Seasonal pre-migratory deposition of fat is well documented in regular seasonal migrants, but is less described for more facultative species. We analyze fat deposits of free-living birds across several taxa of facultative migrants in the songbird subfamily Carduelinae, including house finches (*Haemorhous mexicanus*), American goldfinches (*Spinus tristis*), pine siskins (*Spinus pinus*) and four different North American ecotypes of red crossbills (*Loxia curvirostra*), to evaluate seasonal fat deposition during facultative migratory periods. Our data suggest that the extent of seasonal fat deposits corresponds with migratory tendency in these facultative taxa. Specifically, nomadic red crossbills with a seasonally predictable annual movement demonstrated relatively large seasonal fat deposits coincident with the migratory periods. In contrast, pine siskins, thought to be more variable in timing and initiation of nomadic movements, had smaller peaks in fat deposits during the migratory season, and the partial migrant American goldfinch and the resident house finch showed no peaks coincident with migratory periods. Within the red crossbills, those ecotypes that are closely associated with pine habitats showed larger peaks in fat deposits coincident with autumn migratory periods and had higher wing loading, whereas those ecotypes associated with spruces, Douglas-fir and hemlocks showed larger peaks coincident with spring migratory periods and lower wing loading. We conclude that population averages of fat deposits do reflect facultative migration strategies in these species, as well as the winter thermogenic challenges at the study locations. A difference in seasonal fattening and wing loading among red crossbill ecotypes is consistent with the possibility that they differ in their migratory biology, and we discuss these differences in light of crossbill reproductive schedules and phenologies of different conifer species.

Keywords: annual cycle, Cardeuline, facultative movement, migration, fattening, fueling, finches, nomad

INTRODUCTION

Every year billions of birds migrate to track resources or to mitigate the impact of challenging environmental conditions on survival and reproduction. In most migratory birds, these movements occur largely through long periods of flapping flight (Hedenström, 1993) – an energetically demanding mode of locomotion that can drive the instantaneous costs of migration upward of 10 times the basal metabolic rate for extended periods of time [reviewed in Guglielmo (2018)]. Migratory flight is thus energetically expensive and requires substantial fuel stores to power the sustained muscle activity, especially given that most migratory birds cannot forage while flying.

The energetic demands of flight shape fueling strategies in migrant birds (McWilliams et al., 2004). Fats contain 8 to 10 times as much energy per unit mass as compared to carbohydrates and proteins, in large part due to their hydrophobic nature. Fatty acids are, thus, the primary fuel utilized during migratory flight in birds (Blem, 1980; Ramenofsky, 1990; Bairlein, 2002; Guglielmo, 2018). Birds store some fats directly in working myocytes [i.e., muscle cells; (Marsh, 1984; Napolitano and Ackman, 1990)], but much of the energy required for long distance flight is mobilized from triacylglycerides stored in adipocytes [i.e., fat storage cells; (Blem, 1976; Ramenofsky et al., 1999; Maillet and Weber, 2006)]. Adipocytes are concentrated in a variety of locations in the body, though subcutaneous fat depots are the primary area of storage (Blem, 1976; Maillet and Weber, 2006). The amount of fat stored in extra-muscular deposits is generally thought to reflect the perceived risk of failing to meet energy demands balanced by the risk of increased flight costs or decreased performance when carrying extra fat mass (Videler et al., 1988; McNamara and Houston, 1990; Witter and Cuthill, 1993; Kullberg et al., 1996). For example, birds that have recently experienced unstable food resources (Lehikoinen, 1986; Rogers, 1987; Houston and McNamara, 1993; Bednekoff and Krebs, 1995), high thermoregulatory costs (Helms and Drury, 1960; Newton, 1969; Ekman and Hake, 1990) or long periods of fasting (Kendeigh, 1969; Vincent and Bédard, 1976; Haftorn, 1989) may carry larger fat deposits, whereas high predator density can have the opposite effect (Blem, 1975; Lima, 1986; Pravosudov and Grubb, 1998; Gentle and Gosler, 2001; Lind and Cresswell, 2006; Pascual and Senar, 2015).

Premigratory fat deposits in migrant birds generally reflect the anticipated flight duration prior to refueling opportunities, but can also reflect other aspects of migratory ecology (Odum, 1960; Alerstam and Lindstrom, 1990; Weber et al., 1994). For example, migrants that fly over barriers without refueling opportunities must carry more fat than those that make frequent stopovers for refueling (Blem, 1980; Alerstam and Lindstrom, 1990; Newton, 2008) and migrants may carry more fat than is necessary to provide a buffer for coping with uncertain food resources during stopover or at the breeding grounds (Pettersson and Hasselquist, 1985; Nielsen and Rees, 2013). However, those migrants that face high predation pressure may carry less fat (Alerstam and Lindstrom, 1990) and take longer to replenish fat stores at stopover sites (Cimprich et al., 2005; Schmaljohann and Dierschke, 2005). Further, those birds under pressure for a timely arrival at breeding locations (e.g., high latitude breeders and territorial spring breeders, etc.) may also take more risks during fueling and depart with lower fat stores compared to those that instead take the time to optimize energy costs throughout the journey (Alerstam and Lindstrom, 1990; Wojciechowski et al., 2014). While there exists a relatively rich body of research investigating how ecological and physiological contexts affect fattening in traditional, seasonal to-and-fro migrants [i.e., obligate migrants, reviewed in Blem (1976, 1980), Ramenofsky (1990); Bairlein and Gwinner (1994), Biebach (1996); Jenni and Jenni-Eiermann (1998), Bairlein (2002); McWilliams et al. (2004), Newton (2008); Weber (2009), Guglielmo (2010, 2018), Price (2010), and Pierce and McWilliams (2014)], there are fewer accounts of how fat deposits fluctuate in facultative types of migrations that occur less predictably in space and/or time, as in nomadic and irruptive species and partial migrants.

Facultative migrants may deposit smaller fat stores prior to or during migration than most obligate migrants, though data are much more limited (Alerstam and Lindstrom, 1990). Irruptive and nomadic migrations are thought to occur largely in response to low food resources, often combined with high population density (Newton, 2006a). Yet these movements are often seasonal, as in many boreal seed-eating species (Newton, 2006b; Benkman and Young, 2020; Dawson, 2020), some insectivores [e.g., long-tailed tit (*Aegithalos caudatus*); (Bojarinova and Babushkina, 2015)] and some frugivores [e.g., waxwings (*Bombycilla* spp.); (Witmer, 2020)]. The responsiveness of facultative migrants to dwindling food resources has led some to speculate that facultative migrants may struggle to meet energy demands during migration or to store sufficient fat (Tiainen, 1980; Silverin, 2003), but others challenge that hypothesis given that facultative migrants often carry large fat deposits and maintain high body mass even during mass irruptions when resources should be most limiting (Bojarinova and Babushkina, 2010). Seasonal cycles in fat stores consistent with premigratory fattening have been observed in populations of facultative migrants under both natural conditions (Newton, 1972; Summers et al., 1996; Marquiss and Rae, 2002; Cornelius and Hahn, 2012); but see Alonso and Arizaga (2011) and controlled captive conditions with unlimited food (Berthold and Gwinner, 1978; Hahn, 1995; Babushkina and Bojarinova, 2011; Newton and Dawson, 2011; Cornelius and Hahn, 2012; Robart et al., 2018). Further, the timing of premigratory fattening in the irruptive long-tailed tit and pine siskin (*Spinus pinus)* responds to experimental manipulations of photoperiod in captivity (Babushkina and Bojarinova, 2011; Robart et al., 2018), although apparently not in Eurasian siskins (*Spinus spinus*) (Newton and Dawson, 2011). These studies suggest that even those species with highly flexible migratory behaviors may sometimes utilize endogenous programs and predictive cues to prepare for movements. Given that many facultative migrants do not have consistent migratory routes and destinations across years, fattening may reflect insurance against uncertainty about refueling opportunities. In partial migrant systems, movements may be programmed and consistent, but only in particular populations [e.g., more northerly populations: Linnets (*Linaria cannabina*; Stey et al., 2017); goldcrests (*Regulus*

regulus; Bojarinova et al., 2008) or subsets of populations (e.g., females and juveniles in European blackbirds (*Turdus merula*; Lundberg, 1985; Fudickar et al., 2013) and dark-eyed juncos (*Junco hyemalis*; Holberton, 1993)]. The migratory individuals in these populations show more fattening during the migratory season than do their resident counterparts (Lundberg, 1985; Jahn et al., 2010; Fudickar et al., 2013), but see Holberton (1993). Facultative migrants may thus share some similarities in premigratory physiology with obligate migrants, but exhibit a higher degree of variation at the population level.

Hypotheses and Predictions

We hypothesize that the degree of seasonal fattening observed in a facultative species is associated with variation in timing or prevalence of facultative movements across individuals and populations. We test this hypothesis with long-term datasets from a subfamily of songbirds with variable facultative migration strategies, the cardueline finches, including: house finches (*Haemorhous mexicanus*), American goldfinches (*Spinus tristis*), pine siskins and four different North American ecotypes of red crossbills (*Loxia curvirostra*) to determine if average fat deposits change seasonally and match described migratory patterns.

House finches are resident and experience relatively mild winters at our study locations in California (**Table 1**), thus we predict little or no seasonal fluctuations in fat deposits. American goldfinches are partial migrants at our study locations and the birds that remain in winter experience cold conditions, thus we expect average fat deposits to be larger in wintering goldfinches than in goldfinches captured during the migratory season – which presumably includes migrants intent on traveling variable distances and those birds that remain resident. Pine siskins are irruptive and nomadic with highly variable migratory behavior but with movements centered in fall, peaking in September and October, and in spring, peaking in April and May (Dawson, 2020), although they may move at other times of year less regularly (Palmer, 1968). Moreover migrant and/or transient pine siskins can co-occur with individuals in a resident life cycle stage (e.g., breeding; Granlund et al., 1994; also observed in Eurasian siskins Senar et al., 1992). We therefore predict fat deposits to show high variability during the migratory season in pine siskins and for fat deposits to also be large and comparatively less variable during the cold winter months. Red crossbills are nomadic migrants that commonly initiate movements in May and June to locate newly developing cone crops (Bielefeldt and Rosenfield, 1994; Summers et al., 1996; Marquiss and Rae, 2002; Newton, 2006b; Brady et al., 2019; Gatter and Gatter, 2019; Nothdurft, 2019) and these movements can transition into irruptions later in the summer and fall if no sufficient cone crops are encountered (Newton, 1972, 2006b; Brady et al., 2019; Benkman and Young, 2020). Additional facultative movements commonly occur in the fall in North America (Brady et al., 2019; Benkman and Young, 2020) and have

TABLE 1 | Primary capture locations in the United States for American goldfinches (AMGO), red crossbills (RECR), pine siskins (PISI), and house finches (HOFI).

Location	Latitude (N)	Longitude (W)	Elevation (m)	Species captured
Ypsilanti, Michigan	42°	83°	240	AMGO
Lapeer, Michigan	43°	83°	280	AMGO
Mill Creek, Del Norte County, California	41°	124°	50	RECR
Signal Mountain, Teton County, Wyoming	43°	110°	2300	RECR
Lake Quinault, Grays Harbor County, Washington	47°	123°	58	RECR
Gray's River, Wahkiakum County, Washington	46°	123°	80	RECR
Allen Canyon, Clark County, Washington	45°	122°	40	RECR
Shaw Island, San Juan County, Washington	48°	123°	30	RECR
Devils Table, Yakima County, Washington	47°	121°	1400	RECR
Neah-kah-nie mountain, Tillamook County, Oregon	45°	123°	100	RECR
Camas Creek, Lake County, Oregon	42°	120°	1525	RECR
Fremont National Forest near La Pine, Deschutes County, Oregon	43°	121°	1290	RECR
Deschutes National Forest near Sisters, Oregon	44°	122°	900–1345	RECR, PISI
Eagle Point, Oregon	42°	122°	445	PISI
Mount Hood National Forest, Oregon	45°	121°	745	PISI
Okanogan-Wenatchee National Forest, Chelan County, Washington	48°	121°	760	PISI
Okanogan-Wenatchee National Forest, Kittitas County, Washington	47°	121°	975	PISI
Okanogan-Wenatchee National Forest, Okanogan County, Washington	49°	120°	1360	PISI
Umatilla National Forest, Columbia County, Washington	46°	118°	1850	PISI
Gifford Pinchot National Forest, Skamania County, Washington	46°	122°	1330	PISI
Eagle Rock, California	34°	118°	200	PISI
Jackson, Wyoming	43°	111°	1900	PISI
Moscow, Idaho	47°	117°	790	PISI
Davis, California	39°	122°	10	HOFI
Los Angeles, California	34°	118°	10–50	HOFI

been observed in Europe (Gatter and Gatter, 2019) if seed crops disperse from cones at high rates or birds seek better conditions elsewhere between breeding seasons (Marquiss and Rae, 2002). We therefore predict fat deposits to be high during migratory periods in red crossbills. Winter fattening in crossbills may be complicated by opportunistic winter breeding, if breeding birds carry less fat than nonbreeders as in some other species (Freed, 1981; Krause et al., 2016). We therefore predict migratory fat to exceed winter fat, when evaluated on average across years.

Red crossbills occur as different ecotypes that appear to be adapted morphologically to different conifer species (Benkman, 1993). Differences in conifer seed phenology may therefore drive differences in migratory preparations and morphology in the crossbill ecotypes that feed on them. Thus, we predict ecotypes that forage efficiently on conifers with more consistent annual seed production and retention to show relatively less fattening and ecotypes that forage primarily on species with less annual consistency to show relatively greater fattening. In our study area, lodgepole pine (*Pinus contorta*) has relatively consistent annual production of cone crops in the spring and like other pines has hard cones that open relatively slowly and later, whereas the softer cones of spruces, Douglas firs and hemlocks are more prone to rapid seed shedding (Ruth and Berntsen, 1955; Fowells, 1968; Smith and Balda, 1979; Benkman, 1987; Hahn, 1998; Marquiss and Rae, 2002; Mezquida et al., 2018). We therefore predict that red crossbills will show seasonal peaks in fat deposits in late spring and again in the fall that are more defined in ecotype 3 (closely associated with hemlocks and spruces) and ecotype 4 (closely associated with Douglas-fir and spruces) than in ecotype 2 (closely associated with ponderosa pine) and ecotype 5 (closely associated with lodgepole pine) (Benkman, 1993; Kelsey, 2008). As a further test of whether any ecotype differences in fattening likely reflect differences in migratory strategies, we also examine mass to wing chord quotients – as migrants are expected to have lower wing loading to reduce flight costs (Norberg, 1995; Bowlin and Wikelski, 2008). Thus, we expect that more nomadic ecotypes will have longer wings (and smaller mass to wing chord quotients). We evaluate seasonal patterns in fat deposits in relation to described annual schedules of each species and ecotype to better understand how fat deposits relate to facultative patterns of migration.

MATERIALS AND METHODS

Study Sites

Sites were chosen based on availability of good capture locations within favorable habitat and the presence of target species (**Figure 1** and **Table 1**). American goldfinches were captured at study sites throughout Michigan, with the majority of captures occurring at sites in southeast Michigan. Red crossbills and pine siskins were captured at study sites in Washington, Oregon, Wyoming, and California, with additional pine siskin captures in Idaho. House finches were captured in California, thus house finches were generally captured at more southerly locations than the other species.

Capture and Sampling

Data were collected from adult male and female birds captured at our field sites between 1987 and 2021. During this time we lured 1,352 adult red crossbills, 418 adult pine siskins, 272 adult American goldfinches and 148 adult house finches into mist nets using live decoys or into feeder traps baited with thistle or sunflower seed (**Supplementary Table 1** describes captures by year and month). Juveniles were excluded to avoid age-related complications in timing or propensity of migratory movements in partial migrant species (Prescott and Middleton, 1990), because of potential age-related differences in fat deposits among first year birds, and because it is possible that fattening is limited by foraging ability in juvenile red crossbills during bill development (Jamie M. Cornelius, unpublished observations). We had no pine siskin recaptures during this study and only one house finch recapture (note that approximately 40% of the house finches captured in this study were retained for captive experiments). Twenty-four red crossbills were recaptured once during this study (i.e., approximately 1% of all captures) and 16 American goldfinches were recaptured between one and three times each (i.e., approximately 8% of all captures). We excluded data from recaptured birds given that there were few recaptures and the majority of these occurred within several weeks of the initial capture. For red crossbills, vocalizations were recorded to identify call type either when the bird was approaching the net or when it called upon release (see **Supplementary Table 2** for sample sizes of ecotypes across life cycle stages). Spectrograms were created to assist with assigning type if there was uncertainty. Crossbills that did not vocalize were not assigned a call type and were excluded from type-specific analyses (N = 84). All birds were sexed and aged according to plumage, bill and skull characteristics or reproductive characteristics (Pyle et al., 1997). Birds for which characteristics were not sexually dimorphic at the time of capture (e.g., non-breeding pine siskins) were either molecularly sexed (as in Robart et al., 2018) or categorized as unknown sex and excluded from these analyses.

Wing loading was estimated as the quotient of body mass to wing chord length in lean red crossbills (i.e., individuals with a fat score of 0; see below). Mass was measured by spring scale to the nearest 0.5 g and unflattened wing chord length was measured with dial calipers to the nearest 0.1 mm in red crossbills. While wing shape may be the ideal metric to measure wing loading in migrants (Minias et al., 2015; Buler et al., 2017), wing chord length can predict wing shape in some passerines (Blem, 1975; Lo Valvo et al., 1988), and relates to migratory ecology in many species (Lo Valvo et al., 1988; Marchetti et al., 1995; Boyle, 2008).

Fat Deposits

To assess fueling, we focus on the size of subcutaneous fat deposits. This provides a more direct measure of fuel stores than measures based on body mass, which is known to fluctuate seasonally independently of fat stores (e.g., due to changes in pectoralis muscle and heart size, egg laying or winter thermogenesis) (Ward and Jones, 1977; O'Connor, 1995; Liknes and Swanson, 2011). Subcutaneous fat deposits were scored in

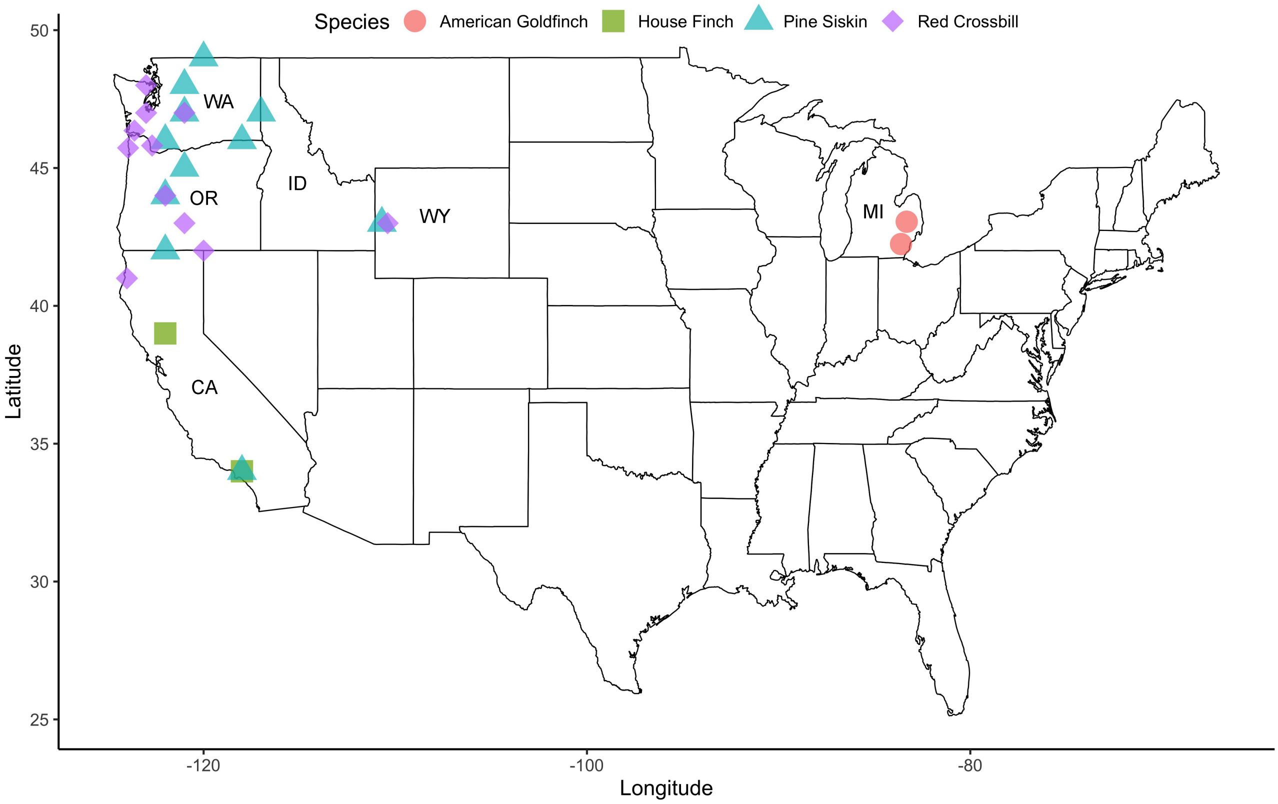


FIGURE 1 | Map of main capture locations for each study species in the United States of America. State borders are shown with states where birds were captured labeled (WA, Washington; OR, Oregon; CA, California; ID, Idaho; WY, Wyoming; MI, Michigan).

the furcular and abdominal cavities on a scale from 0.0 (no fat evident) to 5.0 (gross bulging fat deposits) based on descriptions in Helms and Drury (1960), and used previously in red crossbills and pine siskins specifically (Hahn, 1998; Cornelius and Hahn, 2012; Robart et al., 2018). Briefly, furcular (aka tracheal pit) fat deposits were scored as a 0 if no fat was visible in the pit, a 1 if there was a line of fat at the bottom of the pit, a 2 if fat covered the sides of the pit, a 3 if the fat deposits filled the pit, a 4 if the fat bulged above the pit, and a 5 if fat spilled across the pectoral muscle posterior to the furcular pit. Abdominal fat deposits were scored as a 0 if no fat was visible, a 1 if fat lined the posterior edge of the rib cage, a 2 if fat was visible amongst the intestinal tract, a 3 if fat covered the abdominal cavity, a 4 if fat bulged above the abdominal region and a 5 if fat spilled across the pectoral muscle anterior to the abdominal region. Fat scores from the two regions were summed for analysis. JC and HW were trained in fat scoring during long-term field and captive studies by TH. Other authors were subsequently trained by JC and HW. All participants were trained using the protocol described above; however, different species were generally scored by a subset of the authors on this article: red crossbill data were collected by JC and TH, pine siskin data were collected by HW, AR, BV, and JC. American goldfinch data were collected by JC, DZ., KG, and CN, and house finch data were collected by HW. Although most authors on this article score fat deposits to a much finer resolution than the whole number scale described above (i.e., to the 0.5 or 0.25 resolution), we round fat scores to the nearest whole number to minimize variability between observers. Systematic observer bias was also checked for in our data as described in the section "Statistical Analysis" below and by comparing observer scores of fat deposits collected from photographs of abdominal ($N = 9$) or furcular ($N = 11$) fat deposits of various species.

Fat Deposits in Relation to Timing of Breeding, Molt and Migration

Annual cycles of fat deposits were analyzed by calendar month, but we also assigned months to seasonal life cycle stages of breeding, molt, migration and overwintering based on published species accounts (Badyaev et al., 2020; Benkman and Young, 2020; Dawson, 2020; McGraw and Middleton, 2020; see section "Results"). While sure to introduce error, binning by life cycle stages allows us to condense our data into fewer bins and gives us the necessary power to include interaction terms between life cycle stage and sex in our models (see statistics below). Where possible we used morphological data collected in our study to evaluate the accuracy of the assignment of life stages within the annual schedules of each species (see below)

TABLE 2 | Assignment of capture months to the annual schedule for life cycle stage analysis.

Species	Overwintering	Migration	Breeding	Molt
American goldfinch	January to March	April to May (spring) October to December (fall)	June to July	August to September
House finch	October to April	NA	May to June	July to September
Pine siskin*	December to March	April to May (spring) October to November (fall)	June to August	September
Red crossbill	December	May to June (spring) October to November (fall)	January to April (winter) July to September (summer)	Overlaps

**Note that breeding and migration both commonly occur in pine siskins in the spring. We have identified the migratory period as those months in which movements most commonly occur. See supplement for a more detailed discussion.*

Reproductive timing was estimated using changes in cloacal protuberance (CP) length in males (Bailey, 1952; Wolfson, 1952). CP length increases in response to elevated testosterone from the testes and offers a non-invasive estimate of reproductive condition in male songbirds (Hegner and Wingfield, 1986). CP lengths were averaged and compared across months and breeding was determined to be prevalent in those months where average CP length exceeded the grand mean for CP length across the year. Use of CP length avoids seasonal biases that can occur when using brood patch prevalence if females are required to sit tighter on nests during colder conditions (e.g., winter breeding in red crossbills or early spring breeding in pine siskins).

Replacement of the primary flight feathers proceeds sequentially from wrist to wingtip during the complete, annual pre-basic molt in finches. We scored primary molt progress by recording the outermost feather that was actively growing at the time of capture for each individual, excluding the small tenth primary. The initial stages of primary molt can overlap with breeding in finches and the later stages of molt with migration (Cornelius et al., 2011). Thus, we assigned any months in which there was overlap as either breeding or migration and only assigned as molt those months in which primary molt occurred independently of any other stage.

Statistical Analysis

All statistical analyses were performed in JMP Professional v.14.0 and data and model residuals were analyzed to ensure that model assumptions were not violated. All models included random and fixed effects thus we used linear mixed models. Prior to statistical analyses we checked for inter-observer variability in fat scoring within a given species and month of capture by ANOVA and second by using a least squares model to determine if observer identification or photograph number described variability in fat scores. These tests suggested low inter-individual variability in fat scoring (see results below).

Seasonal Patterns of Fat Deposits Across Species

Seasonal patterns of fat deposits were analyzed by calendar month and by life cycle stage – which were constructed for each species based upon published data and capture data in this study (see section "Results"). To explore seasonal variation in fat deposits we first used a linear mixed model (LMM) fit by restricted maximum likelihood and constructed separately for each species with month (ordinal), sex (categorical), and time of day (continuous) as fixed effects. Fat deposits may vary by year or location in response to weather or other environmental fluctuations. We therefore also included year and location in the model as random effects. For red crossbills and pine siskins we had enough samples when binned by month to also include an interaction term between month and sex, but could not do so for house finches and American goldfinches without saturating the model due to smaller sample sizes. Significant month to month changes in the size of fat deposits were identified from the fixed effect parameter estimates which compared adjacent months (**Supplementary Table 1**) and peaks and valleys in fat deposits across the year were identified using Tukey pairwise comparison of means as the months with the highest or lowest mean fat scores that were significantly different from months with medial fat scores.

Second, we constructed LMMs as described above but replaced month with life cycle stage as the seasonal effect variable (categorical) and included a stage*sex interaction term. Non-significant interaction terms were removed from models (as described below) to recover degrees of freedom and restore conditional effects to main effects. We again identified stages with different fat deposits using fixed effect parameter estimates with overwintering as the comparison group and identified peaks and valleys using Tukey comparison of means (**Supplementary Table 2**).

Seasonal Patterns of Fat Deposits Across Red Crossbill Ecotypes

We did not analyze type differences by month due to sample size limitations that prevented the inclusion of an interaction term between month and type in the model. To analyze seasonal patterns of fat deposits between crossbill types, we constructed a LMM with time of day, crossbill type, life cycle stage and an interaction between type and life cycle stage as fixed effects, and with year as a random effect. Location was not included as a random effect in this model because it is correlated with ecotype. Type-specific seasonal patterns were further compared by identifying peaks and valleys in fat deposits using Tukey comparisons of means.

Wing Loading Among Red Crossbill Ecotypes

Wing loading (i.e., lean body mass / wing chord length) was compared among red crossbill ecotypes using ANOVA, followed by post-hoc Tukey comparisons of means.

RESULTS

Tests of Interobserver Variation in Fat Scoring

We found no significant differences between average observer scores of fat deposits in any species month (ANOVA, $P > 0.05$), except for the month of October in pine siskins ($P < 0.0001$). Fat scores of pine siskins captured in October were lower in 2020 when measured by BV than in 2018 or 2019 when measured by HW. However, average fat scores did not differ between these observers in any other months, indicating that the difference observed in October was more likely due to either year or location variability rather than systematic observer bias. Further, there was very low interindividual variation in the fat scores collected from photographs (average standard deviation = 0.35, range 0 – 0.6). A least squares model found that picture ID predicted fat scores with very high accuracy ($F_{19,129} = 86.1$, $P < 0.0001$, $R^2 = 0.93$), and the addition of observer did not improve the fit ($R^2 = 0.93$) (**Supplementary Figure 1**).

Determination of Life Cycle Stages in Each Species

A detailed description and discussion of the results for the determination of life cycles stages in each species is available in the **Supplementary Material**. Assignment of captures to life cycle stages for all species are summarized in **Table 2**.

Seasonal Patterns of Fat Deposits by Month

Month was a significant effect in models describing fat deposits in American goldfinches ($F_{11,132} = 6.9$, $P < 0.0001$), pine siskins ($F_{9,5.3} = 9.0$, $P = 0.01$), and red crossbills ($F_{11,495} = 10.7$, $P < 0.0001$), but not in house finches ($F_{10,62} = 0.58$, $P = 0.83$) (**Figure 2** and **Supplementary Table 3**). Tukey comparisons of means suggest that fat deposits in American goldfinches peaked during February in the winter and were lowest in the spring, which was similar to the more subtle (and non-significant) seasonal pattern observed in house finches. Pine siskin fat deposits also peaked in winter, were lowest during breeding and were intermediate during the migratory periods. Red crossbills, on the other hand, had peak fat deposits in spring and fall, when movements typically occur, and the smallest fat deposits in the summer and winter, when breeding often occurs. Time of day significantly predicted fat deposits in all species ($P < 0.0001$ for all four species). Fat deposits increased linearly with time of day in all species (linear regression $P < 0.0001$), though variation was high around the best fit line (red crossbill $r^2 = 0.02$; pine siskin $r^2 = 0.03$; house finch $r^2 = 0.18$; American goldfinch $r^2 = 0.08$). The interaction term between sex and month was not significant

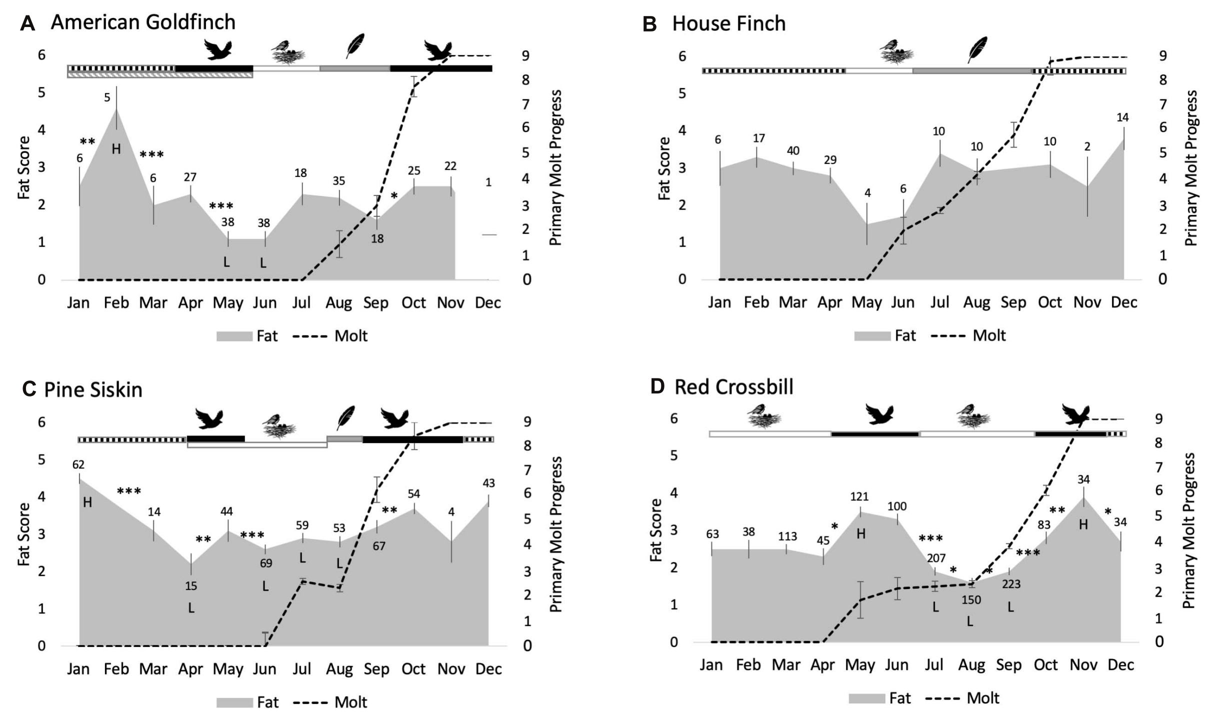


FIGURE 2 | Changes in fat and molt progress across month and life cycle stages in free-living American goldfinches **(A)**, house finches **(B)**, pine siskins **(C)**, and red crossbills **(D)**. Average fat score (gray contour plots) +/– S.E.M. and average number of primaries that have been replaced (dashed line) +/– SEM given for each calendar month. Significant changes in fat score between consecutive months detected by the LMM are indicated by asterisks (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$) and the highest and lowest levels are indicated by the letters H and L (identified by Tukey comparison of means $P < 0.05$). Dominant life cycle stages for each month are shown above the plots, including: overwintering (hatched black bars), migration (black bars), breeding (open bars), prealternate molt (hatched gray), and periods with exclusive primary molt (gray bars). Sample sizes are indicated above each monthly average.

in models explaining fat deposits in red crossbills or pine siskins ($P = 0.2$ and 0.3, respectively) and the interaction term was thus removed from the models. American goldfinches and red crossbills exhibited significant sex differences in fat deposits (American goldfinch $F_{1,218} = 28.9$, $P < 0.0001$; red crossbill $F_{1,1069} = 27.5$; $P < 0.0001$) but house finches and pine siskins did not ($P = 0.8$, and 0.3, respectively). Females had larger fat deposits than males in American goldfinches and red crossbills – though sex alone explained little of the variation (American goldfinch $t_{1,237} = -4.3$; $P < 0.0001$, $r^2 = 0.07$; red crossbill $t_{1,1203} = -6.0$; $P < 0.0001$, $r^2 = 0.03$).

Seasonal Patterns of Fat Deposits Across Species by Life Cycle Stages

Life cycle stage significantly predicted fat deposits in American goldfinches ($F_{4,39} = 4.9$, $P = 0.003$), pine siskins ($F_{4,26} = 8.8$, $P = 0.0001$), and red crossbills ($F_{4,334} = 20.6$, $P < 0.0001$), but not in house finches ($F_{2,97} = 0.65$, $P = 0.52$) (**Supplementary Table 4**). Tukey comparisons of means identified a peak in fat deposits in American goldfinches during the overwintering stage and a valley during the spring migratory and summer breeding stages. Again, this was similar to the more-subtle and non-significant pattern observed in house finches. Pine siskin fat deposits also peaked during the overwintering phase and were lowest in breeding. Fat deposits were intermediate during molt and both migratory stages, but fat deposits in spring migration were indistinguishable from breeding ($P = 0.97$). Red crossbills, on the other hand, had peak fat deposits during spring and fall migration stages and the smallest fat deposits during breeding – which included both summer and winter breeding. The overwintering stage included only the month of December for red crossbills and fat deposits during this stage were intermediate and weakly distinguishable from either migration ($P = 0.06$) or breeding stages ($P = 0.09$). Time of day again significantly predicted fat deposits in all species ($P < 0.0001$ for all four species; patterns are described above in month analysis). The interaction term between sex and life cycle stage was not significant and was removed from all models (American goldfinch $P = 0.3$; house finch $P = 0.1$; pine siskin $P = 0.6$ and red crossbill $P = 0.3$). Sex significantly predicted fat deposits in American goldfinches and red crossbills ($P < 0.0001$ for both species; patterns as described above in month analysis), but not in house finches and pine siskins ($P > 0.5$ for both species).

Seasonal Patterns of Fat Deposits Across Red Crossbill Ecotypes

The interaction between type and life cycle stage significantly predicted fat deposits in red crossbills (life cycle stage*type $F_{12,783} = 5.8$; $P < 0.0001$; type $F_{3,858} = 0.5$; $P = 0.7$; life cycle stage $F_{4,688} = 8.9$; $P < 0.0001$). Fat deposits fluctuated seasonally with a peak during spring migration and a low during breeding in types 3 and 4 ($P < 0.0001$ and $P = 0.0001$; respectively), and the pattern was similar in type 2 – although fat deposits peaked during fall migration, and spring migration fat deposits were not significantly higher than breeding ($P = 0.7$) (**Figure 3**). Type 5s had lower fat deposits during spring migration compared to summer breeding ($P = 0.01$), winter breeding ($P < 0.0001$) and fall migration ($P < 0.0001$), but not overwintering ($P = 0.9$) (**Figure 3**).

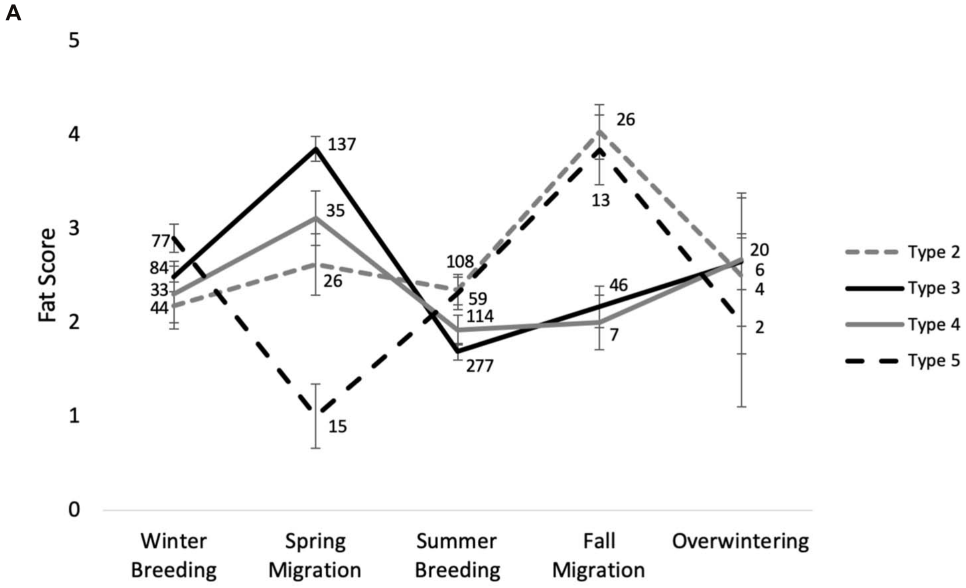
Wing-Loading in Red Crossbill Ecotypes

The quotient of body mass to wing chord length in lean red crossbills varied significantly among types (ANOVA $F_{3,189} = 63.2$, $P < 0.0001$, $r^2 = 0.5$; **Figure 4**). Wing loading was lowest in type 3 ($\bar{x} = 0.319 + 0.002$ SEM, $N = 96$), intermediate in type 4 ($\bar{x} = 0.347 + 0.003$ SEM, $N = 51$) and type 5 ($\bar{x} = 0.349 + 0.006$ SEM, $N = 9$) and highest in type 2 ($\bar{x} = 0.367 + 0.003$ SEM, $N = 34$) (ordered differences by Tukey comparisons of means $P < 0.05$).

DISCUSSION

Seasonal patterns of fat deposits in our long-term data set of four cardueline finch species generally support the hypothesis that population averages reflect different facultative migratory strategies and variable exposure to cold winter climates. The resident house finches captured in regions with mild winter climates can be considered a negative control in this analysis given that we predicted no pre-migratory or thermoregulatory fattening. In agreement, our models detected no significant seasonal variations in fat deposits in this species when analyzed by month or life cycle stage – although it should be noted that our sample sizes are low in spring and may preclude detection of a seasonal low during breeding. Pine siskins and American goldfinches, the two species with perhaps the most variable spring migratory behavior, had larger fat deposits in winter compared to the spring migratory period; whereas red crossbills, arguably the most consistent spring nomad, showed a distinct peak in fat deposits coincident with the late spring migratory period that exceeded winter fat deposits. Although winter breeding may cause adult crossbills to carry less fat as they invest in young (Cornelius et al., 2012; Jamie M. Cornelius, unpublished data), it is notable that fat deposits during the migratory phase in this species were also larger than the fat deposits carried during the cold winter month of December when winter breeding does not occur.

Cold winter temperatures may be both an ultimate and proximate driver of migration for some migrants. One continent-wide metaanalysis of banding records suggests that 11 of 12 partial migrant species migrate away from those areas with the coldest winter temperatures, including pine siskins and house finches (Bonnet-Lebrun et al., 2020). The exception was the American goldfinch – although it tended to overwinter in urban environments in cold places, as many other species do (Bonnet-Lebrun et al., 2020). Pine siskins and American goldfinches may need to carry comparatively more fat than red crossbills in winter due to their smaller size – as can be observed in sparrows of variable body size (Rogers, 2015). Rapidly declining fat deposits from January and February through March and April in these smaller species support that hypothesis. But whereas the partial migrant American goldfinch showed a continued decline in fat deposits through the spring migratory period, the pine siskin



B Within type comparisons

Type 2	B	B	B	A	AB
Type 3	B	A	C	B	B
Type 4	B	A	B	AB	AB
Type 5	AB	C	B	A	ABC

C Between type comparisons

NS 3 > (4 & 2) > 5 (2 & 5) > 3 (2 & 5) > 3 NS

FIGURE 3 | Fat deposits by life cycle stage in free-living red crossbill ecotypes 2, 3, 4, and 5. Fat deposits increased from winter breeding to spring migration in types 3 and 4, but declined or didn't change in pine-associated types 5 and 2, respectively **(A)**. The pattern flipped between summer breeding and fall migration, where pine-associated types showed larger fat deposits than types 3 and 4. There were no type differences in winter. Comparisons of life cycle stages within types **(B)**. Different letters indicate life cycle stages that are significantly different based on Tukey comparison of means $P < 0.05$. Comparison between types within life cycle stages based on Tukey comparison of means $P < 0.05$ **(C)**. Sample sizes given in **(A)**, error bars denote SEM.

showed an elevation in May just after a nadir in fat deposits in April. This increase is consistent with results from captive pine siskins indicating spring migratory preparations of increased fat deposits, body mass, pectoral muscle size and hematocrit, with changes in physiology coincident with an increase in nocturnal migratory restlessness (Robart et al., 2018). Compared with diurnal migration, nocturnal migratory movements may limit the ability to locate foraging sites once underway (Alerstam, 2011) and increased nocturnal migratory behavior may be related to the elevation in fat deposits observed in pine siskins. The timing of migratory preparations in captive pine siskins also differed slightly than observed here, with preparations beginning earlier in captive birds (late March; Robart et al., 2018). This difference may reflect different suites of environmental cues experienced by captive and free-living birds (Calisi and Bentley, 2009).

Pine siskins, American goldfinches, and red crossbills showed fall increases in fat deposits. Pine siskins and goldfinches had a modest increase in the fall relative to the summer nadir, whereas this increase was much more pronounced in red crossbills. This difference between crossbills and siskins may be due to the smaller fat deposits carried by crossbills during breeding, rather than to the absolute levels carried during the fall migratory period, which were more similar between the species. On the other hand, given that pine siskins and goldfinches show more modest increases in fat deposits compared to crossbills during migratory periods, another possibility is that diet differences influence the need for preparatory fattening. Specifically, red crossbills have a more specialized diet compared to pine siskins or American goldfinches (Benkman and Young, 2020; Dawson, 2020; McGraw and Middleton, 2020) and thus may have fewer opportunities to refuel after departure – although insects are likely to play an important role for crossbills during spring migration and in early summer (Thomas P. Hahn and Jamie M. Cornelius, unpublished data).

The increases observed during the migratory periods in these facultative migrants were relatively modest compared to obligate migrants [e.g., the increase in fat score observed in red crossbills during spring migration is half as large as in two similar-sized

North American buntings (Helms and Drury, 1960)]. Pine siskins in particular showed relatively small increases in fat during the spring migratory period, which agrees with other studies in free-living pine siskins where fat deposits were small in birds intercepted at banding stations during apparent migratory events in the fall (Yong and Finch, 2002; Seewagen and Newhouse, 2017). Crossbills nearly doubled fat reserves coincident with the period of fall migration and showed a slightly smaller increase during spring migration in this study. This agrees with data collected on crossbills during an irruption year in Britain that showed small but significant increases in body mass in October and then again just prior to departure in June (Summers et al., 1996), and with captive studies showing similar seasonal changes in mass and fat (Berthold, 1975; Cornelius and Hahn, 2012). Studies on crossbills thought to be more sedentary in pine forests of the Pyrenees found that month was a significant factor in models explaining changes in fat and mass, but post-hoc analysis failed to identify peaks or valleys (Alonso and Arizaga, 2011). Our type-specific analysis here also identified distinctions between crossbills that associate most strongly with spruces, Douglas-fir and hemlocks and crossbills that associate strongly with pines, especially in type 5, which is commonly associated with lodgepole pine.

Fat deposits during winter and summer breeding were not distinct between red crossbill ecotypes in this study but differed in interesting ways during the migratory periods. Fat deposits coincident with the annual nomadic migration in spring were as predicted: type 3 showed the largest increase in fat reserves, followed by type 4 and then type 2, which did not have significantly higher fat during the spring migratory period relative to breeding. This may reflect more consistent nomadic movements in those crossbill types that are associated with conifer species with large annual fluctuations in cone production (Ruth and Berntsen, 1955; Fowells, 1968; Smith and Balda, 1979). Wing loading in red crossbill ecotypes agrees with this hypothesis. Type 3 had the lowest wing loading, consistent with patterns in some other taxa where more migrant individuals (e.g., in partial migrants) or longer-distance migrants have lower wing loading (Pérez-Tris and Tellería, 2001; Nowakowski et al., 2014). Types 2 and 5 had the highest wing loading – suggesting less frequent or shorter-distance migrations and type 5 also showed a unique pattern in fat deposits, with lowest levels occurring during the spring migratory season. The data from types 2 and 5 agree, however, with data from crossbills captured in the Pyrenees that also feed on pines with consistent cone crops (Alonso and Arizaga, 2011), and suggests that crossbills foraging on more consistent food supplies may show less intensive migratory fattening. Alternatively, it is possible that differences in fat deposits during facultative migrations and wing loading reflect the average distance traveled, which is still relatively unknown for different crossbill ecotypes, or refueling options along the way.

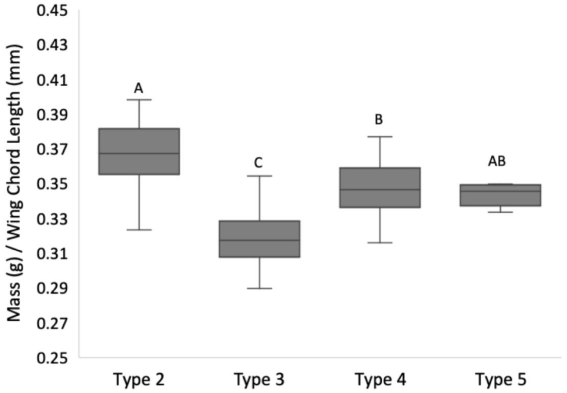


FIGURE 4 | Wing loading in red crossbill ecotypes 2, 3, 4, and 5. The mass to wing chord length quotient in lean red crossbills was lowest in type 3 and highest in type 2. Box plots show inter-quartile range and median lines, whiskers give 95% CI. Box plots with different letters are significantly different based on Tukey comparisons of means ($P < 0.0001$). Sample sizes are 34 type 2, 96 type 3, 51 type 4, and 9 type 5 birds.

Change in fat deposits across crossbill ecotypes did not follow our predictions during the fall migratory period. While fat deposits increased substantially during the fall migratory period in pine-associated crossbill ecotypes 2 and 5, they did not do so in types 3 and 4. One possible explanation is that pine-associated types are most likely to breed in summer on Douglas-fir (*Psuedotsuga menziesii*) and spruces before switching to pine in winter and spring when the seeds are more accessible. This has been documented in Europe (Marquiss and Rae, 2002) and may explain why pine-associated crossbills lay down more fat coincident with periods of fall movements than did the other types. Pine-associated ecotypes do not always move and switch diets, however, as we have observed type 5 crossbills using mostly blue spruce (*Picea pungens*) and Douglas-fir to support breeding in both summer and winter in several different years past in northwestern Wyoming [Jamie M. Cornelius, unpublished data; (Kelsey, 2008)].

American goldfinches are partial migrants and our study site was located toward the northern edge of their wintering range (McGraw and Middleton, 2020). We therefore expected to have captured at least some migrant and some resident birds during migratory months, though it remains possible that our sample was not balanced in this regard, precluding detection of migratory fattening. In many partial migrants the juveniles and females are more migratory and travel further than adult males (Chapman et al., 2011). American goldfinches may differ from this typical pattern in regards to age, but available data suggest that females winter further south than males on average (Prescott and Middleton, 1990). In our study, adult females carried more fat than adult males, but there was no interaction between sex and season, and fattening during spring migration was not apparent in either sex. Our finding of a February peak and a spring nadir in fat deposits is consistent with previous studies of American goldfinches in our study region in southeast MI (Dawson and Marsh, 1986), and with body composition analysis that found peak lipid content and body mass from December through February (Carey et al., 1978). These data support the importance of fat for small-bodied songbirds in winter and suggest that either migratory fueling is low in these diurnal partial migrants, as for other diurnal migrants (Bojarinova et al., 2008;

Stey et al., 2017), or that our sample did not include enough active migrants to detect it.

American goldfinches are also unique in this study in that they are the only species to undergo a prealternate molt that peaks in early spring but is not complete in the population until June or July (Middleton, 1977). Many species separate prealternate molt from migration, presumably to reduce potential energetic trade-offs (Voelker and Rohwer, 1998; Danner et al., 2015). The clear overlap between the prealternate molt and the recorded periods of movement in goldfinches may drive lower fat reserves during the migratory period, as it does in Rusty blackbirds (*Euphagus carolinus*) and Pallas's grasshopper warblers (*Locustella certhiola*) that exhibit variable molt and migration patterns and do not always avoid overlap (Wright et al., 2018; Eilts et al., 2021). However, the late breeding season in American goldfinches may also allow for a longer, slower prealternate molt (Middleton, 1977), which may reduce the costs of molt-migration overlap.

House finches are considered the least migratory species in our study sample and we captured them in regions with mild winter climates. Our finding that fat deposits did not change seasonally is consistent with a previous study of body composition in house finches in southern California (Dawson et al., 1983). However, similar examinations of house finches in areas with much colder winter climates have found seasonal elevations of body fat in the winter (Dawson et al., 1983; O'Connor, 1995). Thus, the absence of seasonal peaks in fat deposits, associated with either migratory or wintering stages, likely reflects both the lack of migratory behavior among house finches in California and a relatively mild climate or consistent food availability in the study locations.

In conclusion, we found support for seasonal fattening in facultative migrant finches coincident with migratory timing and consistent with annual and population-level variations observed in migratory behavior. We also identified ecotype-specific variation in the seasonal patterns of fattening in red crossbills that associate with different conifer species – suggesting that the degree of fattening may relate to probability of relocating to find new cone crops in a given season and/or the anticipated distance of nomadic movements. Remote tracking in this species and in other facultative migrant species will be illuminating in teasing apart these hypotheses further. Geolocators are particularly useful for demonstrating residence in more sedentary or site faithful populations of finches because they require recapture (Alonso et al., 2017) and radiotelemetry-based network systems such as the motus wildlife tracking network could be utilized in some localities where towers are installed (Taylor et al., 2017). The further miniaturization of satellite trackers, however, could be revolutionary for understanding patterns of movement in facultative migrants like Cardueline finches and such systems will allow for a much more accurate understanding of how premigratory preparations relate to actual migratory behavior in these facultative species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Data are available at ScholarsArchive@OSU at https://ir.library.oregonstate.edu/concern/datasets/9g54xr201.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees at Oregon State University, Eastern Michigan University, University of Washington, University of California-Davis, Loyola Marymount University, and Washington State University.

AUTHOR CONTRIBUTIONS

JC and HW conceived of the study. JC, TH, AR, BV, DZ, KG, CN, and HW collected the data. JC performed the statistical analysis and wrote the first draft of the manuscript. JC and BV generated figures. All authors contributed to manuscript editing and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.691808/full#supplementary-material

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Evaporative Water Loss and Stopover Behavior in Three Passerine Bird Species During Autumn Migration

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Migratory birds are often not specifically adapted to arid conditions, yet several species travel across deserts during their journeys, and often have more or less short stopovers there. We investigated whether differences in thermoregulatory mechanisms, specifically evaporative cooling, explain the different behavior of three passerine species while stopping over in the Negev desert, Israel. We measured cutaneous water loss (CWL) under ambient conditions and the temperature of panting onset in an experimental setup. In addition, we performed behavioral observations of birds at a stopover site where we manipulated water availability. Blackcaps had slightly higher CWL at relatively low temperatures than Willow Warblers and Lesser Whitethroats. When considered relative to total body mass, however, Willow Warblers had the highest CWL of the three species. Blackcaps started panting at lower ambient temperature than the other two species. Taken together, these results suggest that Willow Warblers are the most efficient in cooling their body, possibly with the cost of needing to regain water by actively foraging during their staging. Lesser Whitethroats had a similar pattern, which was reflected in their slightly higher levels of activity and drinking behavior when water was available. However, in general the behavior of migratory species was not affected by the availability of water, and they were observed drinking rather rarely. Our results indicate that differences in thermoregulatory mechanisms might be at the basis of the evolution of different stopover strategies of migratory birds while crossing arid areas such as deserts.

Keywords: Negev, thermoregulation, panting, cutaneous water loss, water availability

INTRODUCTION

Thermoregulation in desert habitats is a challenge for endothermic animals. When ambient temperatures (T_a) exceed body temperature (T_b), they need to dissipate excess heat produced endogenously as well as gained from the environment to regulate T_b. Consequently, several behavioral and physiological strategies have evolved among desert-living animals to cope with these conditions. Behavioral strategies include avoiding exposure to excessive heat by e.g., living in burrows, or being active when temperatures are not at their maximum (Yousef and Dill, 1971; Abáigar et al., 2018; Whitford and Duval, 2020). The only way to dissipate body heat at $T_a > T_b$ is to evaporate water from body surfaces (Dawson, 1982), but since water is scarce in the desert, several mechanisms have evolved in desert species to reduce evaporative water loss and avoid dehydration (King and Farner, 1961; Louw, 1993; Williams and Tieleman, 2005; Donald and Pannabecker, 2015). In birds, evaporative cooling is primarily achieved through cutaneous water loss (CWL) and respiratory water loss (RWL) (Whittow, 1986; Williams and Tieleman, 2005; McKechnie and Wolf, 2019). The proportion of CWL and RWL to total evaporative water loss (TEWL) varies among species, but it is generally around 50% at moderate T_a's (Wolf and Walsberg, 1996; Tieleman and Williams, 2002; Ro and Williams, 2010). When T_a approaches or exceeds T_b, birds increase both cutaneous and respiratory water loss, but the latter to a greater extent (Wolf and Walsberg, 1996; Tieleman and Williams, 2002; Wojciechowski et al., 2021). With the exception of Columbiforms (McKechnie and Wolf, 2004), respiratory water loss is the main avenue of water loss when a bird is exposed to heat stress (Wolf and Walsberg, 1996; Tieleman and Williams, 2002; Wojciechowski et al., 2021). Birds experiencing high rates of TEWL may have difficulties to keep their body adequately hydrated while maintaining body temperature below critical hyperthermia (Webster, 1991). Thus, it becomes an obvious challenge, especially for diurnal birds, to reduce TEWL while preventing lethal hyperthermia (McKechnie and Wolf, 2010; Conradie et al., 2020). Tolerance of hyperthermia is the primary physiological mechanism allowing for water conservation in heat exposed birds (Calder and King, 1974; Weathers, 1981; McKechnie and Wolf, 2019). It has been shown that desert birds have a lower TEWL than species from mesic areas (Williams, 1996). CWL is reduced through a specific lipid composition of the skin's stratum corneum, which makes it less permeable to endogenous water (Tieleman and Williams, 2002; Haugen, 2003; Haugen et al., 2003; Muñoz-Garcia and Williams, 2011; Champagne et al., 2012). A reduction of RWL in desert birds as a mechanism of water conservation has been discussed (Williams and Tieleman, 2005) and was observed in zebra finches (*Taeniopygia guttata*) acclimated to limited water availability (Wojciechowski et al., 2021). However, in the Kalahari Desert, bird species relying on food as their only water source started panting at a higher temperature and have overall higher evaporative scope when compared with species that drink water (Smit et al., 2016; Czenze et al., 2020). The ambient temperature of panting onset potentially sets the time boundaries in which a bird can remain active during the day in a desert environment, while avoiding the excessive water loss induced by RWL for thermoregulation at higher T_a (Weathers, 1981; Smit et al., 2016; Pattinson et al., 2020).

Besides local desert specialists, many temperate-zone birds stage in the desert for a relatively short time during migration. In the European-African migration system, around 2.1 billion passerine and near-passerine birds cross the Sahara Desert during their autumn migration to reach their wintering grounds (Moreau, 1972; Hahn et al., 2009). These birds presumably did not evolve specific adaptations to the extremely arid climate conditions of the desert, yet most songbirds do not overfly the Sahara in one non-stop flight, but rather fly at night and stop over during the day (Biebach, 1985; Bairlein, 1988; Schmaljohann et al., 2007). Notably, many species avoid long stopovers in the desert and prolong their flights as much as possible (Adamík et al., 2016; Jiguet et al., 2019; Malmiga et al., 2021). Nonetheless, these *trans*-Saharan migrants are known to perform longer stopovers prior to the desert crossing during autumn (Yosef and Chernetsov, 2005). Specifically, many migrating birds stop in the Negev desert which is located at the northern edge of the Sahara Desert (Moreau, 1972; Yosef and Chernetsov, 2005). Despite being defined as an arid environment (Goldreich, 2012), the Negev is the last potential stopover area before the Sahara Desert crossing during autumn. Birds that stage at the desert edge are staging for longer periods, accumulating large amounts of fat to facilitate the cross-desert travel (Piersma, 1998; Schaub and Jenni, 2000; Wojciechowski et al., 2014). Observational studies showed that the behavior at desert stopover sites varies among species (Jenni-Eiermann et al., 2011; Arizaga et al., 2013; Hama et al., 2013; Maggini et al., 2015). These differences are likely associated with adaptations to arid habitats: while xerophilic species (e.g., many Mediterranean species) spend longer stopovers in the desert and effectively refuel, mesophilic species usually avoid long stopovers and spend rather short time on the ground resting in the shade (Jenni-Eiermann et al., 2011).

The difference in stopover patterns between xerophilic and mesophilic migratory species may result from differences in their ability to maintain water balance. Foraging behavior and fat accumulation of migratory Blackcaps (*Sylvia atricapilla*, a mesophilic species) at a desert stopover site was influenced by water availability, while this was not the case in Lesser Whitethroats (*Sylvia curruca*, a xerophilic species) (Sapir et al., 2004; Tsurim et al., 2008). In addition, Lesser Whitethroats were able to accumulate energy stores in a similar fashion in a wide range of habitats, while in Blackcaps this was restricted to a habitat offering ideal feeding conditions, such as high amounts of lipid-rich fruits (Sapir et al., 2004; Domer et al., 2018).

In this study, we aimed at understanding the physiological mechanisms underlying the different behavior of birds at desert stopovers in relation to water availability and their ability to maintain water balance. We hypothesized that *trans*-Saharan migratory songbird species adjust their stopover behavior in the desert to minimize the risk of dehydration. We assumed that species better adapted to arid conditions face lower risk of dehydration. In particular, we predicted that species actively refueling at a stopover in the Negev have a lower surface-specific CWL and express a higher temperature threshold for panting onset than species which spend their stopover resting in the shade. We also predicted that an experimental manipulation of water availability leads to a change in foraging behavior, especially in species with low refueling rates, inducing an increase in refueling when water was available. To test these predictions, we quantified foraging and drinking behavior, CWL, and temperature of panting onset in Blackcaps and Lesser Whitethroats, taking advantage of the previous knowledge of their different stopover strategies during autumn migration in Israel (Sapir et al., 2004; Tsurim et al., 2008). We also measured CWL and panting onset in the Willow Warbler (*Phylloscopus trochilus*). This species is regularly observed to actively forage at desert stopover sites in spring (Jenni-Eiermann et al., 2011; Maggini et al., 2015). If indeed differences in thermoregulatory CWL underlie differences in stopover behavior among species, Blackcaps should have a higher CWL and a lower temperature of panting onset than the other two species. The augmented availability of drinking water should increase foraging activity in all species, but more so in Blackcaps.

MATERIALS AND METHODS

Study Site

We collected data during the autumns of 2017 and 2018 at two stopover sites in the Negev Desert, Israel (**Figure 1**). In 2017,

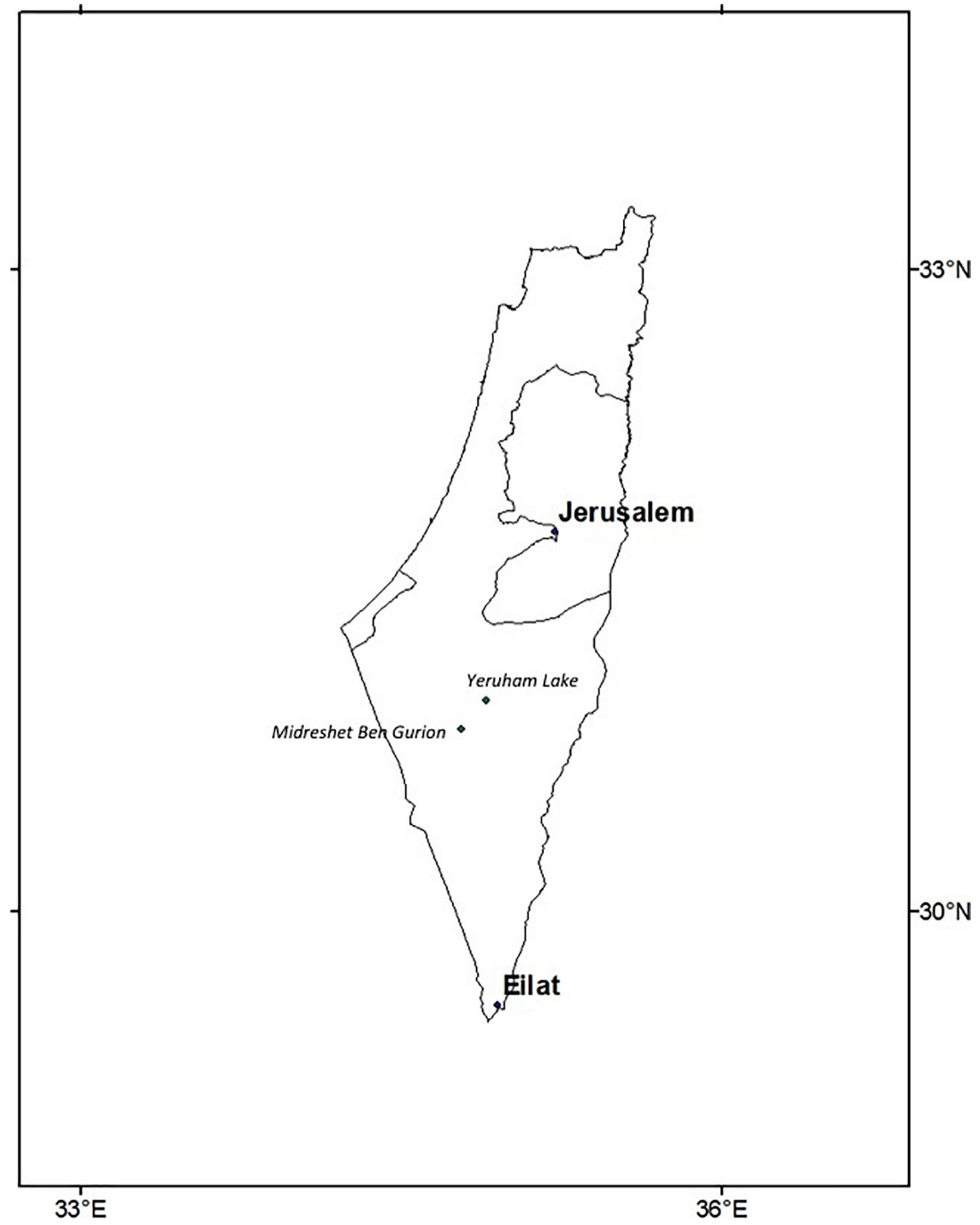

FIGURE 1 | The location of the two field sites in Israel. The study was conducted at Lake Yeruham in 2017, and at Midreshet Ben-Gurion in 2018.

our study site was Lake Yeruham (30°59′ N, 34°53′ E), a large artificial waterbody in the northern Negev. The vegetation was dominated by *Phragmites australis, Tamarix aphylla,* and *Tamarix nilotica* as well as *Xanthium strumarium*. At some distance from the lakeshore, the most common plant species were *Acacia raddiana, Atriplex halimus, Morus sp.,* and *P. australis.* During autumn 2018 we collected data at Midreshet Ben-Gurion (30°51′ N, 34°46′ E), approximately 19 km south of the 2017 site. This site includes an experimental mixed *Pistacia* tree plantation (1.7 ha) which is populated mainly with two species, *Pistacia lentiscus* and *Pistacia chinensis*, in addition to *Acacia saligna* and *A. halimus.*

Study Species

We studied Blackcaps, Lesser Whitethroats and Willow Warblers. These three species differ in body mass and size (Shirihai and Svensson, 2018; own data in **Table 1**) and have similar breeding origins (Yosef Kiat, Israeli Bird Ringing Center, unpubl. data). Blackcaps and Willow Warblers winter mainly in forested areas, although they are both highly flexible in their habitat selection (Snow et al., 1997; Shirihai et al., 2001; Sapir et al., 2004; Baker, 2010). Even though both species use similar habitats, they have different refueling strategies: migratory Blackcaps rely mainly on pre-departure fattening rather than refueling in desert oases especially during autumn migration (Izhaki and Maitav, 1998; Sapir et al., 2004; Tsurim et al., 2008; Wojciechowski et al., 2014), and Willow Warblers spend more time foraging en route and carry relatively low fuel loads (Jenni-Eiermann et al., 2011; Haran and Izhaki, 2013; Maggini et al., 2015). The preferred winter habitats of Lesser Whitethroats are savannas with scattered trees or thornbush savanna (Snow et al., 1997; Shirihai et al., 2001; Sapir et al., 2004). The refueling strategy of Lesser Whitethroats is not thoroughly studied, but they show positive refueling rates in the region of our study (Sapir et al., 2004).

Field CWL Measurements

To quantify differences in evaporative water loss among bird species we conducted CWL measurements at Midreshet Ben-Gurion during autumn 2018. Birds were captured using mist nets. Captures started at 5:30 AM and were stopped when ambient temperature exceeded 30°C. Captured birds were marked with aluminum leg ring, after which the length of the folded wing and body mass were measured using international standards (Bairlein, 1995). Then, the birds were kept in a cotton bag in a shaded location until CWL measurements were taken. At the beginning of each measurement, we recorded time, air temperature, and air humidity using a mobile digital thermo-hygrometer (NeKan EU). Relative humidity (RH, in %) was transformed to absolute humidity (AH, in g H_20 m^{-3}) using the formula AH = C × P_w/T, where C is a constant of 2.16679 (gK J^{-1}), P_w is the partial pressure of water vapor in Pa, and T is the temperature in K. We calculated P_w as P_{ws} (saturation water vapor pressure in hPa) × RH. P_{ws} was obtained using the formula P_{ws} = A × 10 ^ [m × T/(T + T_n)], where A, m and T_n are constants (respectively, 6.1164341, 7.591386, and 240.7263 for temperatures between −20°C and +50°C), and T was the temperature in °C.

TABLE 1 | Mean body mass and wing length (mean ± 1 SD) of the three species measured in this study.

Species	Body mass (g)	Wing length (mm)
Blackcap (*S. atricapilla*, *n* = 205)	17.9 ± 2.5	76.7 ± 2.0
Lesser Whitethroat (*S. curruca*, *n* = 118)	12.7 ± 1.7	67.0 ± 2.1
Willow Warbler (*P. trochilus*, *n* = 134)	9.0 ± 1.1	67.2 ± 3.2

The sample size refers to all birds measured with respirometry in 2017 and with the vapometer in 2018.
Sample sizes differ from later analyses because of the exclusion of birds showing no changepoint in the analysis of panting onset.

The measurement was made by an experimenter who exposed at least 50 mm^2 of the lateral body apterium under the bird's left wing, while wearing latex gloves to avoid moistening of the bird's skin by human sweat. Then, five subsequent measurements of surface-specific CWL were taken using a factory-calibrated VapoMeter^{TM} (Delfin Technologies, Ltd., Kuopio, Finland, hereafter: vapometer; du Plessis et al., 2013). We used the small adapter of the vapometer, which covers 16 mm^2 of skin (Muñoz-Garcia and Williams, 2007; Muñoz-Garcia et al., 2012), and each measurement took about 20–30 s. The whole procedure lasted between 7 and 10 min, and immediately afterward the birds were released at the site of capture. Visual fat score (Kaiser, 1993) was assessed after the vapometer measurement to avoid blowing moist air onto the bird's skin.

Temperature of Panting Onset

This part of the study was conducted at Lake Yeruham during autumn 2017. Birds were captured using mist nets from 5:30 AM until at least five individuals of the study species were caught. Captured birds were marked and measured as described above.

We used a flow-through respirometry protocol to measure the temperature of panting onset. A bird was placed into a two-compartment respirometry chamber (Wolf and Walsberg, 1996) with which we intended to measure CWL and RWL separately. The upper section of the chamber (head) was 8.75 l and the lower section (body) was 11.25 l. Due to persistent baselining problems, we failed to measure the absolute values for evaporative water loss and O_2 consumption. However, the output allowed us to record the temperature of panting onset using the sliding heating protocol (see below). For this analysis we used data from the upper section of the chamber (head compartment).

We performed the measurements using a sliding heating protocol. The respirometry chamber with the bird was placed inside a temperature-controlled box (35 l MobiCool G35). We started the measurement with the flow rate through the chamber set to 3.3 l min^{-1} for 15 min to enable a quick washout of the humidity accumulated in the chamber during the positioning of the bird. After the washout period, we decreased the flow to 0.66 l min^{-1} and started the heating of the chamber. Temperature in the temperature-controlled box was monitored continuously by a custom-made temperature logger (precision: ±0.1°C) attached inside the respirometry chamber. The starting temperature of the measurements was 31.0 ± 3.4°C and progressively increased during the procedure at a rate of ∼0.3°C min^{-1}. We continued the measurement for at least 5 min after noticing a sharp increase in excurrent air humidity in the upper chamber, which was a clear indication of the onset of panting. On average, the measurement of one bird took one hour and the average temperature at the end of the experiment was 39.3 ± 1.6°C. The bird was immediately released at the end of the trial, after being provided with water. We did not perform visual observations of bird behavior during these measurements.

Behavioral Responses to Changes in Water Availability

We experimentally examined the effect of water availability on the foraging and drinking behavior of staging birds at Midreshet Ben-Gurion in 2018. We manipulated water availability at the same study site in which bird captures with mist netting took place (see CWL measurements). We created five drinking puddles of water using plastic sheets and fresh tap water. The puddles were placed in the shadow of large bushes 6–12 m from the closest mist net, with the exception of one puddle which was directly below a net. The plastic sheets were covered with soil before the puddles were filled to achieve a more natural setting. The puddles were filled and emptied alternately in a 5-day rhythm. The water regime change was always conducted at 10 PM. In total, water was provided on 13 days (two full 5-day periods and one truncated period at the end of the season), while the puddles were dry on 11 days (two full 5-day periods and one day before the start of the experiment). One additional artificial water body (150 × 70 cm) was present at the site. It was built of concrete and had vertical walls. This reservoir was most likely not a suitable drinking place for birds, and none were ever observed to use it for drinking or bathing. However, we covered it with wire mesh (0.5 cm mesh size) when the experimental ponds were dry and uncovered when water was provided at the experimental puddles.

We searched for Blackcaps and Lesser Whitethroats along a transect in the plantation. One observer walked the transect each day at 09:00, 11:00, 13:00, and 17:00 between September 5 and September 29 (excluding September 10 and September 16). The transect was approximately 1090 m long and its coverage took 45 min. The observer scanned vegetational structures (bushes and trees) along a defined path for at least 2 min during each round. When a bird was encountered, we classified its behavior in one of the following four categories: (1) moving, (2) foraging, (3) resting, and (4) grooming. We observed each individual for 30–300 s and assigned a single behavior to each bird. When more than one behavior was expressed, we chose the behavior that lasted longer during the observation. Moving was defined as occasions when the bird was jumping or flying around. If during the movement the bird was seen pecking or eating at least once during the observation, we classified its behavior as foraging, because we assumed that movement could be related to food-searching. Resting was defined as cases when the bird just perched on a branch without undertaking any other activity. Grooming was defined as the bird taking care of its plumage during the observation time. It never occurred that a bird was foraging and resting/grooming during one observation sequence.

In addition to these observations, we set trail cameras (Cuddeback Long Range IR) at 80–130 cm distance from the edge of each artificial puddle. The cameras were attached to sticks 15–20 cm above the ground. They were set to take a picture and start a video recording for 30 s following every movement trigger and were active throughout the study period (including when the puddles were dry). We analyzed all pictures and videos and recorded all bird species present and the duration of their visit. Time and date were also recorded.

DATA ANALYSIS

Field CWL Measurements

In a first step of the analysis, we explored the factors that explained variation in CWL. We used a linear mixed-effects model with the surface-specific CWL ($g\ m^{-2}\ h^{-1}$, measured with the vapometer) as a dependent variable. We used the average of the three lowest vapometer measurements as the response variable because the measured values decreased gradually from the first to the fifth measurement, reaching a plateau between the third and fifth. Since ambient temperature as well as air humidity influence CWL (Wolf and Walsberg, 1996; Tieleman and Williams, 2002; Gerson et al., 2014; Champagne et al., 2016), we included them as independent variables in the analysis, together with water in the puddles (no/yes), ordinal date, waiting time in the bag (CWL measurement time - extraction time from the net), and size-corrected body mass. Size-corrected body mass was calculated as: [(body mass/length of the folded wing) × (length of the folded wing)]. We used species as a random intercept to be able to detect within-species variation in CWL in relation to body mass. We eliminated non-significant predictor variables in a stepwise backward procedure, checking after every step that model deviance was not affected (Zuur et al., 2009). We examined all combinations of predictor variables used in the models for multicollinearity using scatterplot matrices and correlation coefficients (Pearson's r). All calculated $|r|$ - values were below 0.7, which is considered to be a suitable indicator value above which multicollinearity severely distorts model estimations (Dormann et al., 2013). The model was tested for major violations of model assumptions by evaluating diagnostic plots (Zuur et al., 2009). After identifying the most influential parameter (which was T_a, see section "Results"), we tested for interspecific differences using ANCOVA, with surface-specific CWL as a dependent variable, T_a as a continuous independent variable, species as a categorical independent factor, and the species × T_a interaction. The assumptions for ANCOVA were checked and met.

To determine the variability in total CWL in the three species, we modeled the slopes and intercepts for each species as obtained from the ANCOVA model. We obtained hourly ambient temperature data from the Israel Meteorology Service station located in Kibbutz Sde-Boker, approximately 3 km north-east of the Midreshet Ben-Gurion site. In both years September temperatures ranged between 14.0 and 36.5°C, which overlaps with the range of temperatures at which bird CWL was measured. Using the equations describing the relationship between CWL and T_a, we calculated changes in total CWL (g H_2O h^{-1}). Body surface area was calculated as As (cm^2) = 10 body mass $(g)^{0.667}$ (Walsberg and King, 1978). To simplify the approach, we assumed that CWL changes during the day only in relation to changes in T_a. We do realize that this approach is very simplistic, yet it provides information on the ecologically relevant species differences in CWL. Daily CWL was obtained by integrating the total CWL over a given day. Finally, we simulated changes in CWL using ambient temperature for days in September 2017 and 2018. Integrated total CWL was compared between species using a repeated ANOVA on ranks. We then compared CWL as a percentage of total body mass lost during the day in the three study species.

Temperature of Panting Onset

We used the absolute humidity values from the upper chamber as a proxy for RWL. We matched the temperature measurements in the temperature-controlled box with the humidity measurements from the upper chamber such that our dataset included data for every minute. We excluded the first 25 min (15 min washout + 10 min equilibrium establishment) from the analysis. We used breakpoint analysis to identify the temperature at which a sharp increase in chamber humidity, an indication of panting, took place. We determined the breakpoint in the curve between the 26th minute of the measurement procedure and its end using the "segmented" function provided by the "segmented" package in R (Muggeo, 2009) and used this breakpoint as the temperature of panting onset for every individual. Sometimes the linear increase in RWL after the onset of panting became exponential toward the end of the measurements. We removed such points prior to the calculation of the segmented regression due to their high leverage on the estimate. Birds failing to show a clear breakpoint were not included in the analysis (12 out of 88; 3 blackcaps, 4 lesser whitethroats, and 5 willow warblers). We compared the temperatures of panting onset between species using a one-way ANOVA after we ascertained that the normality assumption was met, as was the homogeneity of variances between the samples (Levene's Test from medians: $F_{2,73} = 0.473$, $p = 0.625$). We used a Tukey HSD *post hoc* test to examine species-specific differences at a 95% confidence level.

Behavioral Responses to Changes in Water Availability

The probability of finding moving or foraging birds is consistently higher than for resting birds. To deal with this bias, we analyzed the relationship between daily captured and observed individuals in Blackcaps and Lesser Whitethroats. We ran a generalized linear model (GLM) with a Poisson error family, with the number of birds observed as a dependent variable, and the number of birds captured and the species as independent variables. The model fit was checked by visually inspecting diagnostic plots (Faraway, 2016). We cannot exclude that we observed some individuals multiple times, but we considered these observations as independent since we were not able to differentiate among individuals.

All analyses were performed with R 4.0.2 (R Core Team, 2020) within the RStudio IDE (version 1.4.1717).

RESULTS

Field CWL Measurements

We obtained 360 CWL measurements from the three study species (Blackcap: $n = 165$, Lesser Whitethroat: $n = 89$, Willow Warbler: $n = 108$). The results of the mixed-effects model are shown in **Table 2**. The variables retained in the model were T_a, AH, date, water in the puddles, and size-corrected body mass. The effect sizes of AH and date were small and were considered irrelevant, and in addition, the low t-value (<2) for AH indicated that this effect was not significant. T_a had a positive effect on surface-specific CWL, while the availability of water and size-corrected body mass were negatively correlated to surface-specific CWL (**Table 2** and **Figure 2**). The ANCOVA confirmed the positive effect of T_a on surface-specific CWL (**Table 3**). There were also significant differences between species, with Blackcaps having higher surface-specific CWL than Lesser Whitethroats and Willow Warblers (**Table 3**). This was mostly due to higher values at low temperatures, as confirmed by the significant difference in the slope of surface-specific CWL in relation to T_a (**Table 3** and **Figure 3**).

Daily absolute CWL simulated by our model equaled: 2.025 ± 0.085 g H_2O day^{-1} in Blackcaps, 1.520 ± 0.083 g H_2O day^{-1} in Lesser Whitethroats, and 1.230 ± 0.072 g H_2O day^{-1} in Willow Warblers. Expressed as a percentage of body mass, Blackcaps would lose 11.31 ± 0.47%, Lesser Whitethroats 11.96 ± 0.65%, and Willow Warblers 13.71 ± 0.80% of body mass daily by cutaneous evaporation only (**Figure 4**). These differences among species were statistically significant (rm ANOVA on ranks, $\chi^2 = 120$, $df = 2$, $p < 0.001$).

Temperature of Panting Onset

We estimated the temperature of panting onset of 76 birds (Blackcap: $n = 30$, Lesser Whitethroat: $n = 25$, Willow Warbler: $n = 21$). It differed significantly between the three study

TABLE 2 | Results of a linear mixed-effects model with surface-specific CWL as dependent variable.

Fixed effects:

	Estimate	Std. error	*t*-value
Intercept	−4.066	3.888	−1.046
T_a	0.416	0.023	17.844
Ambient humidity	0.046	0.042	1.106
Date	0.028	0.013	2.123
Water (yes)	−0.487	0.188	−2.590
Size-corrected body mass	−0.135	0.044	−3.098

Random effects:

		Variance	Std. dev.
Species	*Intercept*	0.752	0.867
Residual		2.964	1.722

N = 360 observations of three species.

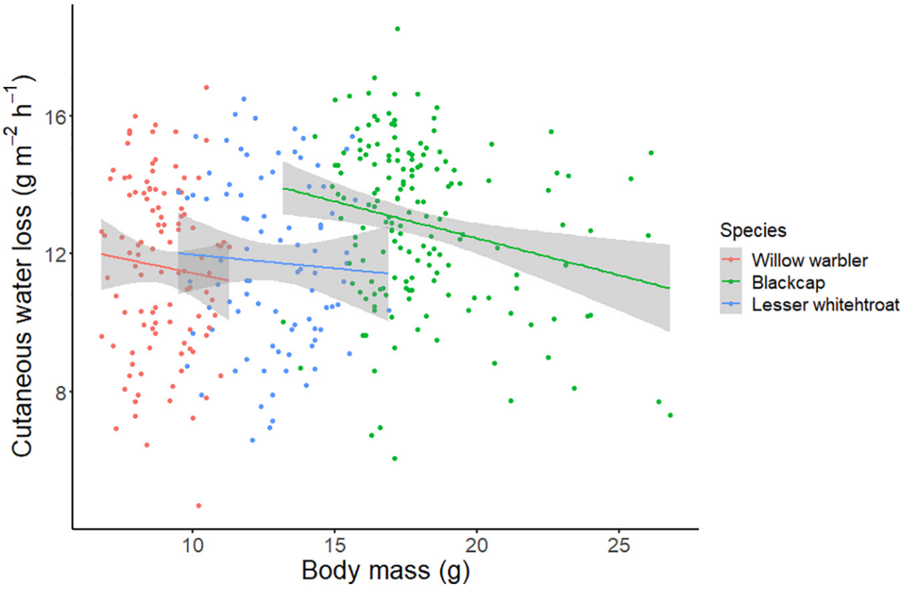

FIGURE 2 | Relationship between body mass and surface-specific CWL in the three study species. In all three species, there is a negative relationship between body mass and CWL.

species (one-way ANOVA: $F_{2,73}$ = 5.137, p = 0.008, **Figure 5**). Blackcaps started panting at a lower temperature (mean ± 1 SD: 36.5°C ± 1.6°C) than Lesser Whitethroats (37.6°C ± 1.5°C; Tukey HSD *post hoc* Test: adj. p = 0.048) and Willow Warblers (37.9°C ± 1.8°C; Tukey HSD *post hoc* Test: adj. p = 0.020). Lesser Whitethroats and Willow Warblers did not differ in their temperature of panting onset (adj. p = 0.804).

Behavioral Responses to Water

During autumn 2018 we captured 181 Blackcaps, 89 Lesser Whitethroats and 133 Willow Warblers. During 90 transects (45 min each, totaling 67.5 h) we observed 26 Blackcaps and 83 Lesser Whitethroats. The number of birds observed increased on average by 0.12 ± 0.04 with every captured bird (GLM: z = 3.02, p = 0.003), and the slope of the increase was not different between species (GLM, interaction term species × birds captured: estimate = 0.01 ± 0.06, z = 0.14, p = 0.888). Significantly more Lesser Whitethroats were observed along the transects than Blackcaps (GLM, main effect of species: estimate = 1.69 ± 0.51, z = 3.29, p = 0.001).

TABLE 3 | Results of the ANCOVA with surface-specific CWL as a dependent variable.

Coefficients

	Estimate	Std. error	*t* value	*p*
Intercept	0.637	0.998	0.638	0.524
T_a	0.461	0.041	11.162	< 0.001
Species (Blackcap)	3.386	1.310	2.585	0.010
Species (L. Whitethr.)	0.726	1.472	0.493	0.622
T_a × Species (Blackcap)	−0.119	0.052	−2.268	0.024
T_a × species (L. Whitethr.)	−0.041	0.060	−0.680	0.497

Residual standard error was 1.757 on 356 degrees of freedom.
Adj. $R^2 = 0.505$, $F_{5,356} = 74.68$, $p < 0.001$.
In this table, the Willow Warbler is used as a reference category for the effects of species.

Most birds were observed moving or foraging in the vegetation (Blackcap: 92.6%; Lesser Whitethroat: 95.1%). The remaining birds were resting (**Figure 6**). The proportion of foraging and moving birds did not differ significantly between days with water availability and days with no water (Chi-square test: χ^2 = 0.050, df = 1, p = 0.822). Comfort behavior and resting were excluded from the test due to small sample sizes or lack thereof.

In 2018 we recorded 143 individuals from 13 bird species at the artificial puddles. The most abundant were resident bird species, while only a few migratory birds occurred at the ponds for drinking. Of our focal species, four Blackcaps, nine Lesser Whitethroats and four Willow Warblers were observed at the puddles. Mean duration of stay at the puddle did not differ among the three species (one-way ANOVA: $F_{2,13}$ = 0.342, p = 0.717, **Table 4**).

DISCUSSION

We found partial support to our prediction that our three study species would have different adaptations to arid conditions. Blackcaps had slightly higher surface-specific CWL than Lesser Whitethroats and Willow Warblers, but this difference was mostly due to higher CWL at T_a's well below T_b. When extrapolated over the whole body surface, the species with the highest overall CWL were also the ones showing higher activity levels, which is against our expectations. However, as predicted, the onset of panting occurred at lower T_a in Blackcaps compared

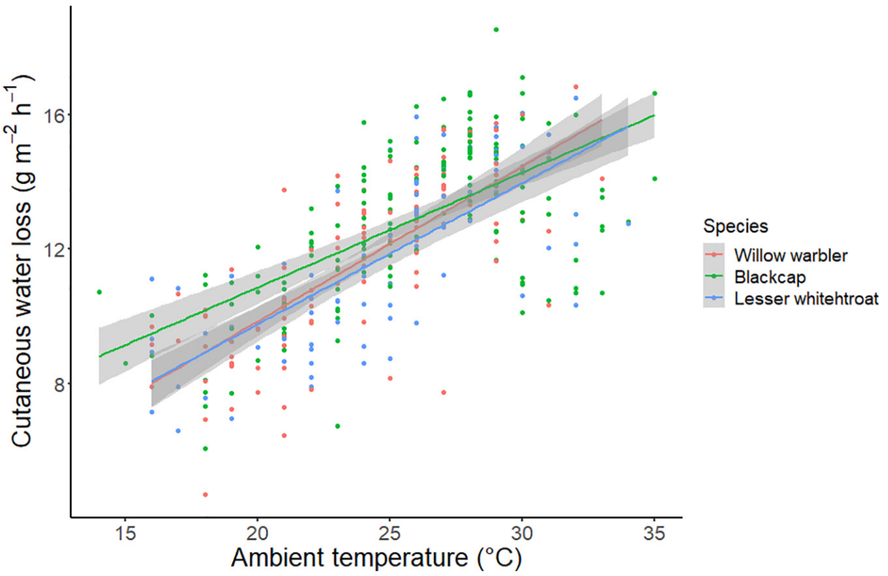

FIGURE 3 | Relationship between T_a and surface-specific CWL in the three study species. Blackcaps (green) had significantly higher overall CWL than the other two species, as a result of higher values at low T_a.

to the other two species. The availability of water did not seem to influence the birds' activity. Taken together, these results do not support the hypothesis that the activity of birds at a stopover site would be associated with the risk of dehydration. However, physiological constraints related to water can help explain the birds' behavior.

The measured maximum CWL ranged between 34 and 36 mg H_2O cm^{-2} day^{-1}, and were within the range of 12 temperate zone passerine bird species (21.8–35.8 mg H_2O cm^{-2} day^{-1}) measured in dry conditions (Ro and Williams, 2010). Our simulated data show that this represents a daily CWL of 11–13% of the total body mass of the focal species. Though we have no data on RWL, we can assume that this would have similar values, because in passerines, at T_a's below the panting threshold, there is an approximate 50:50 ratio of CWL and RWL (Tieleman and Williams, 2002; Muñoz-Garcia and Williams, 2005). Therefore, assuming that a bird would sit completely still in a shaded area during a whole day, its TEWL would be in the range of 20–26% of body mass, depending on the species. This by far exceeds the 11% dehydration threshold for the maintenance of coordination in small passerines (Wolf and Walsberg, 1996). In the bird species measured so far, total body water in healthy individuals is about 60–65% of body mass (Hughes et al., 1987; Ellis and Jehl, 1991; Speakman, 1997). Our predicted water loss is therefore in the range of 31–43% of the total body water. This implies that birds must replenish their water reserves to avoid death by dehydration. Foraging activity might expose the animals to higher T_a and, possibly, direct sunlight. This, together with an increase in metabolic heat production due to muscular work would sharpen the need for evaporative cooling and result in even higher water loss.

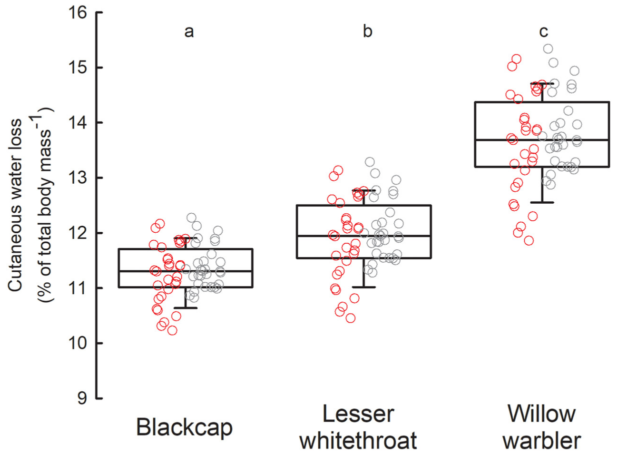

FIGURE 4 | Daily CWL of the three study species as simulated from our empirical data. Each point represents a single day in September 2017 (red dots) and 2018 (gray dots). The values are given as percentage of body mass (taking the average body mass for each species as estimated in this project, **Table 1**). Different letters indicate significant difference between medians (Tukey test: $p < 0.001$).

While part of the lost body water might be produced metabolically (Morrison, 1953), the main avenue for obtaining additional water for most bird species is food, especially when no drinking water is available. Some species are even known not to drink when surface water is available (Smit et al., 2016; Czenze et al., 2020), and for these species it is imperative to obtain their water through foraging. Despite the percentage of active birds observed was similar for Lesser Whitethroats and Blackcaps, the proportion of observed to trapped birds was much higher for Lesser Whitethroats. This suggests that inactive Blackcaps were likely underrepresented in our observation study,

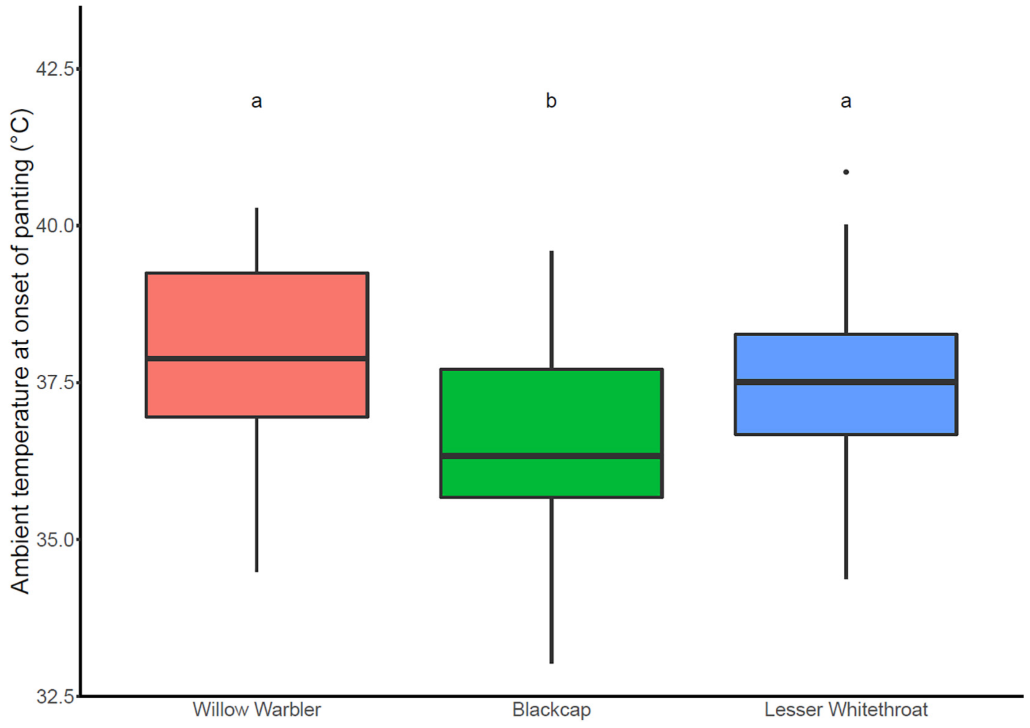

FIGURE 5 | Temperature of panting onset in the three study species. Species marked with the same letters (a, b) were not significantly different. Willow Warbler (orange): *n* = 21; Blackcap (green): *n* = 30; Lesser Whitethroat (blue): *n* = 25.

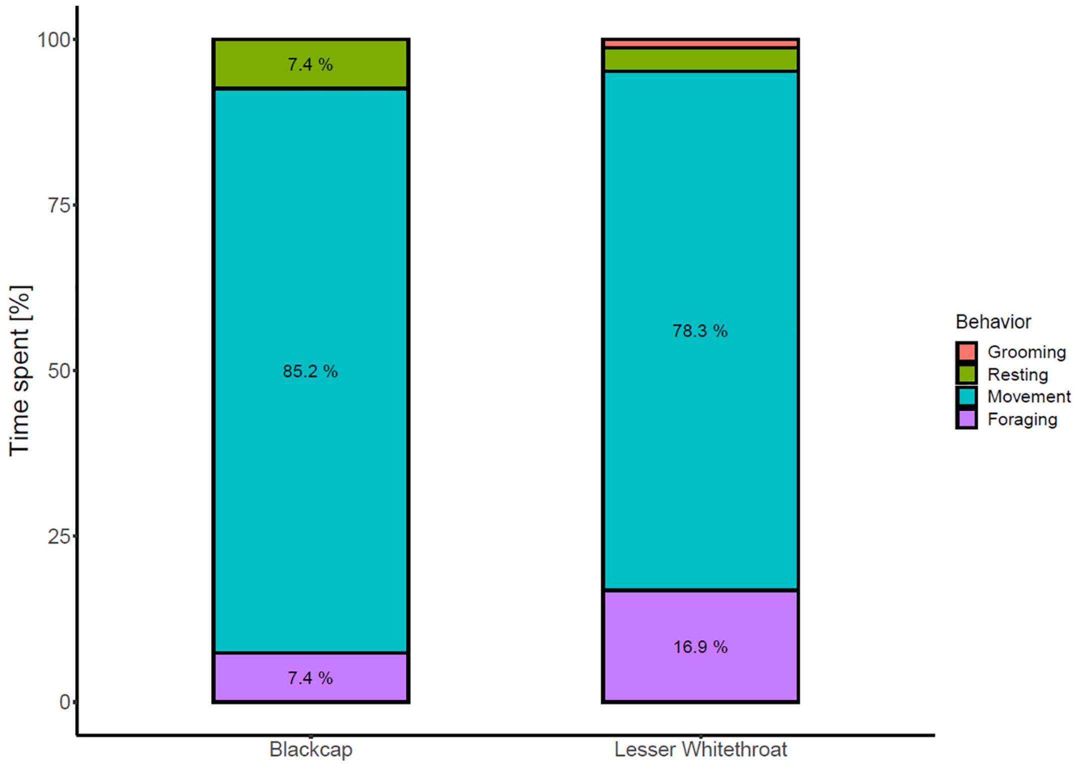

FIGURE 6 | Behavior of Blackcaps and Lesser Whitethroats at Midreshet Ben-Gurion in September 2018. Behavioral observations were not performed on Willow Warblers. Blackcap: *n* = 26; Lesser Whitethroat: *n* = 83. In Lesser Whitethroats, values for resting and grooming behavior were 3.6 and 1.2%, respectively.

TABLE 4 | Number of birds recorded and mean duration of stay (±SD, only for species with $N > 3$) at the artificial puddles during the study in 2018.

Species	Water		Total captures	Mean duration (sec)
	Yes	No		
European Turtle Dove *Streptopelia turtur*	2	2	1	
Red-backed Shrike *Lanius collurio*	1	0	18	
White-spectacled Bulbul *Pycnonotus xanthopygos**	51	0	13	24.4 ± 7.9
Willow Warbler *Phylloscopus trochilus*	**4**	**0**	**122**	**12.3 ± 10.9**
Blackcap *Sylvia atricapilla*	**4**	**0**	**143**	**18.5 ± 8.6**
Common Whitethroat *Sylvia communis*	5	0	10	21.0 ± 6.7
Eastern Orphean Warbler *Sylvia crassirostris*	1	0	12	
Lesser Whitethroat *Sylvia curruca*	**9**	**0**	**89**	**15.3 ± 11.4**
Arabian Babbler *Turdoides squamiceps**	14	1	0	16.7 ± 9.6
Spotted Flycatcher *Muscicapa striata*	1	0	11	
Rufous-tailed Scrub Robin *Cercotrichas galactotes*	30	17	3	22.1 ± 9.5
Ortolan Bunting *Emberiza hortulana*	1	0	1	

*Target species are outlined in bold and resident species are marked with *.*
The total number of captures refers to the period 05.09.2018–27.09.2018 and is given as a proxy for the abundance of the species.
However, species of large size might be underrepresented because mist nets are not designed for capture of these species.

because of lower detectability. This suggests that, overall, Lesser Whitethroats are more active than Blackcaps, which can be associated with the higher percentage of body water that they lose through CWL. Unfortunately, we have no data on the activity of Willow Warblers, but previous studies conducted in the Sahara Desert showed that they are also actively foraging during stopovers (Jenni-Eiermann et al., 2011; Maggini et al., 2015). Intriguingly, these differences do not seem to have arisen by different adaptations of the skin membranes among species (as shown e.g., in larks and sparrows, Tieleman and Williams, 2002; Muñoz-Garcia et al., 2012), since the surface-specific rate of CWL was fairly comparable among species. It is possible that a difference would be observable during spring migration, since Blackcaps spend the winter in more mesic habitats than Lesser Whitethroats (Snow et al., 1997; Shirihai et al., 2001) and their skin membranes might be accordingly flexibly adjusted to the arid conditions at the wintering grounds (Muñoz-Garcia et al., 2008). The higher percentage of water loss in the Willow Warbler could be an indication of higher evaporative cooling efficiency (the rate of heat loss over heat production). As a result, this species may afford higher activity and exposure to higher T_a, provided that the amount of water lost does not exceed a threshold above which dehydration would pose a death threat (Albright et al., 2017; Conradie et al., 2020).

The main behavioral and physiological indication of the risk of dehydration at high ambient temperatures is the onset of panting (Pattinson et al., 2020). The results of this study confirmed the prediction that Blackcaps have a lower temperature of panting onset than Willow Warblers and Lesser Whitethroats. The mean values we measured in the three species (Blackcap: 36.5°C, Lesser Whitethroat: 37.6°C, Willow Warbler: 37.9°C) were in the range of temperatures (31.3°C–46°C, mean: 39.3°C) at which 50% of the individuals from 33 different Kalahari Desert bird species were observed to begin panting (Smit et al., 2016). This result provides a promising avenue to explain the evolution of the different use of stopover sites in migratory species. It also indicates that behavioral characteristics of response to heat could be relevant for sites outside the desert as well. It has to be noted that larger species usually initiate heat dissipation behaviors (e.g., panting and wing drooping) at lower temperatures than smaller species due to their smaller surface to volume-ratio (Weathers, 1981; Smit et al., 2016; Pattinson et al., 2020). This could be an explanation for the observation in our study. In addition, different adaptations to optimize heat loss in arid conditions, such as the ability to tolerate high body temperatures during facultative hyperthermia (Tieleman and Williams, 1999; Smit et al., 2013, 2016; Nilsson et al., 2016), the dependence on wing-drooping as alternative heat dissipation behavior (Smit et al., 2016; Wojciechowski et al., 2021) or the reduction of metabolic rate (Williams and Tieleman, 2005; Wojciechowski et al., 2021) may also influence the temperature of panting onset. These factors clearly indicate new avenues for further research investigating the response of different species to dehydrating conditions.

Interestingly, surface-specific CWL was negatively correlated with body mass in all three species studied. Since body mass is largely affected by fat stores in migratory birds, this provides an indication that subcutaneous fat may be directly or indirectly related to cutaneous evaporation. However, the mechanistic processes involved are still unknown yet. Surface-specific CWL values were also lower when water was available in the puddles. This difference was relatively small (~ 0.5 g m^{-2} h^{-1}) but statistically significant. This may suggest that birds with slightly lower CWL were more likely to be captured on days where water was available. This is somewhat counterintuitive, because we would have expected birds with higher water losses to be more motivated to replenish their water content by drinking or foraging more actively. A possible explanation might include a combination of the effects of body condition (heavier birds – the ones with lower CWL – could be the ones that are more active when water is available) and diet. Birds that consume drier food are more affected by the lack of water (Mizrahy et al., 2011). These birds might be taking advantage of the availability of surface water.

Our study integrates physiological and behavioral data for better explaining differences in stopover ecology among three migratory species that were about to cross a wide ecological barrier, the Sahara Desert, on their way from the temperate breeding areas to their sub-Saharan over-wintering grounds. Our comparison between captured and observed birds showed that Lesser Whitethroats are observed far more often than Blackcaps at the study site, despite the lower number of captures. This confirms that Blackcaps spend more time resting in deep foliage than Lesser Whitethroats at this autumn stopover site in Israel. Despite simulating Sapir et al.'s (2004) study closely, our data do not suggest that the availability of drinking water would affect the behavior of Blackcaps by inducing increased foraging as they were only observed drinking at the artificial puddles rarely, and proportionally less than Lesser Whitethroats. However, Midreshet Ben-Gurion was recently found to act as an unordinary stopover site. While many passerines during autumn migration are attracted to it, most of them abandon it during their first morning (Domer et al., 2018). Given this information, the behavioral part of this study should be treated accordingly, as birds will actively forage only a few days after landing once they fully restored their digestive capacity (Gannes, 2002; McWilliams and Karasov, 2005).

The results of this study suggest that evaporative water loss provides an important physiological background that might have played a crucial role in the evolution of different desert-crossing strategies in small *trans*-Saharan migrants. While some species rely on en route refueling to cross the Sahara, others gain the necessary amounts of fat prior to the desert crossing (Jenni-Eiermann et al., 2011; Arizaga et al., 2013; Hama et al., 2013). Both strategies are successful and enable billions of birds to cross this large ecological barrier twice a year (Moreau, 1972; Hahn et al., 2009). However, the populations of many migratory bird species of the Old World are declining, and especially *trans*-Saharan migrants do so to a greater extent than resident or short-distance migrants (Vickery et al., 2014). Climate change leads to more arid conditions for the whole African continent (Nicholson et al., 2018), potentially imposing constraints on birds that use both refueling strategies before, during and after crossing the desert. While species which rely on pre-departure fattening might be confronted with the energetic challenge of carrying even higher fat loads in order to be able to cross an enlarged desert barrier, species which refuel en route might experience more difficulties to find suitable oases for efficient fat accumulation.

This study provides a first step toward identifying potential physiological mechanisms that constrain the birds' behavior during a challenging phase of their migration. However, many open questions on the physiological mechanisms involved in the evolution of different refueling strategies and their species-specific consequences for the entire migration process remain to be addressed. We argue that explaining the behavior of migratory birds through physiological adaptations should be addressed through a mechanistic approach by applying comparative and experimental studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://phaidra.vetmeduni.ac.at/o:669.

ETHICS STATEMENT

The animal study was reviewed and approved by Israel Nature and National Parks Authority.

AUTHOR CONTRIBUTIONS

IM, ES, and NS conceived the study. BP, BW, DB, and YZ performed fieldwork. BP, BW, MW, and IM performed data analysis. BP, BW, AD, MW, and IM wrote the manuscript. YZ, ES, and NS provided logistical support. All authors read and approved the final version of the manuscript.

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Testing Predictions of Optimal Migration Theory in Migratory Bats

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Optimal migration theory is a framework used to evaluate trade-offs associated with migratory strategies. Two strategies frequently considered by migration theory are time minimizing, whereby migration is completed as quickly as possible, and energy minimizing, whereby migration is completed as energetically efficiently as possible. Despite extensive literature dedicated to generating analytical predictions about these migratory strategies, identifying appropriate study systems to empirically test predictions is difficult. Theoretical predictions that compare migratory strategies are qualitative, and empirical tests require that both time-minimizers and energy-minimizers are present in the same population; spring migrating silver-haired (*Lasionycteris noctivagans*) and hoary bats (*Lasiurus cinereus*) provide such a system. As both species mate in the fall, spring-migrating males are thought to be energy-minimizers while females benefit from early arrival to summering grounds, and are thought to be time-minimizers. Thermoregulatory expression also varies between species during spring migration, as female silver-haired bats and males of both species use torpor while female hoary bats, which implant embryos earlier, are thought to avoid torpor use which would delay pregnancy. Based on optimal migration theory, we predicted that female silver-haired bats and hoary bats would have increased fuel loads relative to males and the difference between fuel loads of male and female hoary bats would be greater than the difference between male and female silver-haired bats. We also predicted that females of both species would have a greater stopover foraging proclivity and/or assimilate nutrients at a greater rate than males. We then empirically tested our predictions using quantitative magnetic resonance to measure fuel load, $\delta^{13}C$ isotope breath signature analysis to assess foraging, and ^{13}C–labeled glycine to provide an indicator of nutrient assimilation rate. Optimal migration theory predictions of fuel load were supported, but field observations did not support the predicted refueling mechanisms, and alternatively suggested a reliance on increased fuel loads via carry-over effects. This research is the first to validate a migration theory prediction in a system of both time and energy minimizers and uses novel methodological approaches to uncover underlying mechanisms of migratory stopover use.

Keywords: optimal migration theory, thermoregulation, stopover ecology, physiological ecology, bats

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INTRODUCTION

Billions of birds and bats migrate annually to take advantage of seasonally abundant resources. Many species interrupt migratory movements with periodic stopovers which are critical to the overall success of migration as they provide opportunities to rest, refuel, and seek refuge. Despite their importance, stopovers often account for the majority of time and energy costs associated with migration (Hedenström and Alerstam, 1997; Wikelski et al., 2003) and in some cases could account for greater than 90% of the total energetic costs of migration (Clerc and McGuire, 2021). Thus, to fully understand migration ecology it is necessary to consider the consequences of stopover behavior.

One approach used to generate predictions of stopover behavior is optimal migration theory, which uses an optimization modeling framework to theoretically frame the adaptive value associated with varied migratory strategies (Alerstam and Lindström, 1990). The goal of optimal migration theory is to determine behavioral expressions that maximize fitness (Alerstam and Lindström, 1990). However, because fitness is difficult to quantify during migration, alternative currencies (i.e., fitness surrogates), such as time, energy, or predation risk, are considered to generate predictions about stopover behaviors (Alerstam and Lindström, 1990; Hedenström and Alerstam, 1995, 1997; Lank et al., 2003; Schmaljohann and Dierschke, 2005; Jonker et al., 2010). For example, time may be an appropriate currency if it is adaptive for a migrant to reach their destination as quickly as possible (i.e., time minimizer) (Hedenström and Alerstam, 1997; Hedenström, 2008). Conversely, energy may be an appropriate currency if a migrant experiences selective pressure to reduce the overall energetic costs of migration (i.e., energy minimizer) (Hedenström and Alerstam, 1997). One of the foundational predictions of optimal migration theory is that time minimizers, relative to energy minimizers, will accumulate a larger fuel load at stopover in response to variable fuel deposition rates (Alerstam and Lindström, 1990; Hedenström and Alerstam, 1997).

The mechanism by which migrants manipulate fuel deposition rates and achieve different stopover departure fuel loads depends on the migration theory currency being optimized. To minimize the total time of migration, migrants are expected to increase the net energy accumulation rate at stopover (Hedenström and Alerstam, 1995; Piersma and van Gils, 2011). Conversely, to achieve the minimum total energy of migration, migrants are predicted to maximize the stopover foraging gain ratio (i.e., maximize the gross amount of energy ingested per unit of energy expended; Hedenström and Alerstam, 1995; Hedenström, 2008; Piersma and van Gils, 2011). Given differences in how individuals treat foraging opportunities, time minimizers are predicted to maximize net intake rates by having greater foraging intensity and/or greater nutrient assimilation capacity (rate at which ingested food is converted into fuel) relative to energy minimizers (Houston and McNamara, 2013).

Predictions of stopover departure fuel load, and the mechanisms by which migrants manipulate fuel load at stopover, become more complex when considering the influence of varied thermoregulatory expressions. Many migrants employ a homeothermic pattern of temperature regulation to keep body temperature relatively constant even outside the thermoneutral zone (i.e., the range of ambient temperature (T_a) at which body temperature regulation is achieved without regulatory changes in metabolic heat production; IUPS Thermal Commission, 2003). In particular, as T_a drops below the thermoneutral zone, homeotherms increase their metabolic rate to produce excess heat (e.g., Cryan and Wolf, 2003; Wikelski et al., 2003; for a full treatment of different thermoregulatory strategies in migration see Clerc and McGuire, 2021). For homeotherms, a large proportion of the energetic cost associated with stopover can be attributed to the rapid use of endogenous fuel to remain euthermic via shivering and non-shivering thermogenesis (Hedenström and Alerstam, 1997; Wikelski et al., 2003). However, many species have the capacity to use a heterothermic pattern of temperature regulation during migration and may express hypometabolic states ranging from deep torpor heterothermy (Carpenter et al., 1993; Cryan and Wolf, 2003; McGuire et al., 2014) to facultative hypothermia (or rest-time hypothermia) (McKechnie and Lovegrove, 2002; Wojciechowski and Pinshow, 2009; Carere et al., 2010). When migrants enter hypometabolic states at stopover during non-active periods, they offset anywhere from 5 to >90% of the energetic cost associated with remaining euthermic, increasing fuel deposition rates (Carpenter et al., 1993; McGuire et al., 2014; Baloun and Guglielmo, 2019) and resulting in predictions of reduced stopover departure fuel load (Clerc and McGuire, 2021). Thus differences in thermoregulatory expressions introduce previously unconsidered mechanisms by which fuel loads can be manipulated (i.e., reducing resting costs rather than increasing intake rates) and can change the magnitude of the difference between predicted differences in stopover departure fuel loads between time and energy minimizers.

Empirically comparing migration theory predictions presents a challenge because predictions are qualitative and thus need to be tested in systems where relative differences can be detected. Identifying populations of migrants with seasonally unique migratory strategies is difficult, because for many species all individuals are under similar selective pressures (e.g., arrive to summering grounds to establish territories and breed). However, due to temporal differences in reproductive investment, spring migrating bats present an opportunity to test migration theory predictions under natural conditions. Silver-haired bats (*Lasionycteris noctivagans*) and hoary bats (*Lasiurus cinereus*) are examples of migratory bat species that exhibit mismatched reproductive investment between sexes. Copulation typically occurs in autumn (Racey, 1982; Cryan et al., 2012). Females store sperm throughout the winter and begin spring migration either continuing to store sperm or in early stages of gestation. Female hoary bat reproductive phenology is approximately 1–month advanced relative to silver-haired bats, and by mid-May (spring migration) hoary bats may have well developed embryos while silver-haired bats are just implanting (Druecker, 1972). In spring, females must arrive at summering grounds such that they can identify and occupy high quality maternity roosts prior to parturition and subsequent pup-rearing, with enough time for newborn pups to develop before autumn migration. Conversely, males incur the majority of reproductive costs during autumn (Druecker, 1972), and there is unlikely strong selection to arrive to summering grounds more quickly as it would provide little fitness advantage. Thus, it should be adaptive for females to complete migration as quickly as possible (time minimizers) and males to reduce the overall energetic cost of spring migration (energy minimizers).

In addition to sex differences in optimization currencies, hoary and silver-haired bats differ in thermoregulatory expressions throughout spring migration. Males of both species use torpor at stopover (Cryan and Wolf, 2003; McGuire et al., 2014), as do female silver-haired bats (Jonasson and Guglielmo, 2019). However, female hoary bats avoid torpor use during migration (Cryan and Wolf, 2003). Although capable of using torpor while pregnant (Willis et al., 2006), female hoary bats forgo torpor use during the early stages of gestation, presumably as a strategy to ensure embryo development is not delayed (Cryan and Wolf, 2003; Willis et al., 2006). In contrast, Jonasson and Guglielmo (2019) found that silver-haired bats used torpor during spring migration. Torpor use by female silver-haired bats may be possible because of delayed implantation relative to hoary bats (Druecker, 1972), thus allowing torpor expression without trading-off embryo development.

These differences in reproductive phenology, across sex and species, lead to three migrant "types": time-minimizing homeotherms (female hoary bats), time-minimizing heterotherms (female silver-haired bats), and energy-minimizing heterotherms (male silver-haired and hoary bats). Our objective was to use optimal migration theory to generate field predictions relating differences in fuel load and foraging tactic for the three migrant "types" and empirically field test the resulting predictions during the spring migratory period.

MATERIALS AND METHODS

Study System

We captured silver-haired bats and hoary bats during the month of May from 2016 to 2018 at two sites in the Sandia-Manzano Mountains, New Mexico, United States. We captured most bats in 2016 and 2017 in David Canyon, Bernalillo County (34.99°N, 106.36°W). In 2018 we split capture time between David Canyon and San Pedro Creek (Bernalillo and Sandoval counties; 35.22°N 106.30°W) which is approximately 25 km North of David Canyon. Unfortunately, little is known about the origins and final destinations of the bats that use this stopover site. For hoary bats, historic capture rates are highest during the spring migratory period and then decrease throughout the summer (Findley and Jones, 1964; Druecker, 1972). Based on collection records, the site is likely near the beginning of the migratory journey as winter records of silver-haired bats and hoary bats exist for southern New Mexico (Cryan, 2003). Winter behavior is mostly unknown for both species, though at least some hoary bats remain semi-active throughout winter (Weller et al., 2016). Specific destinations are unknown, though hoary bats and silver-haired bats summer throughout a broad range of the continental interior (Cryan, 2003; Cryan et al., 2014; Hayes et al., 2015).

We captured bats in mist nets and placed them into cloth bags prior to processing. We recorded sex, body mass (± 0.1 g), and forearm length (± 0.1 mm) of each bat. We measured sunset T_a at our capture site for each capture occasion.

Generating Field Predictions

Based on the natural history of silver-haired and hoary bats, we hypothesized that females minimize time and males minimize the total energy cost of migration (for a full treatment of time and total energy cost of migration currencies, see Hedenström and Alerstam, 1997). We parameterized the currencies by sex and species to generate qualitative predictions of optimal fuel load (**Figure 1**). To determine how sensitive prediction outcomes were to changes in currency parameters we compared predictions across a range of mean ambient temperature, stopover settling costs, and daily energetic expenditures. While quantitative predictions varied, relative qualitative differences between males and females and the magnitude of the difference in fuel load between male and female hoary bats compared to the difference in fuel load between male and female silver-haired bats were robust. Qualitative predictions only changed when stopover ambient temperature, which ultimately drives differences in thermoregulatory related energy costs, approached the lower critical temperature of the thermoneutral zone (for a full treatment of the influence of thermoregulatory strategies on optimal migration theory predictions of fuel load see Clerc and McGuire, 2021). For these species the lower critical temperature is between 27 and 30°C (Cryan and Wolf, 2003; McGuire et al., 2014). We did not observe temperatures that high at our field site during this study, thus we believe our parameter values are reasonable and our qualitative predictions to be sound.

Stopover Settling Costs and Daily Energy Expenditure

Predictions of optimal fuel load hinge on stopover settling costs (time and energy spent finding appropriate roosting and foraging locations upon arrival at the stopover site) as well as the fuel deposition rate achieved at stopover. We generated values of settling costs and daily energy expenditure following the methods of Hedenström and Alerstam (1997) and Clerc and McGuire (2021) by combining reasonable assumptions of bat stopover behavior with allometrically scaled mass-specific estimates of metabolic costs assuming mean daytime $T_a = 15°C$ (mean daytime T_a at our field site). We further assumed bats arrive at a stopover site near dawn and required 20 min to find a suitable day roost, upon which they rested in the roost for the daytime period (14.5 h) before emerging the following evening resulting in a settling time cost of $t_0 = 0.6$ days for all groups. Settling fuel load costs (expressed as a ratio of fat mass and lean mass) varied among groups due to differences in mass-specific metabolic rates and thermoregulatory mode with greatly reduced settling for groups using torpor. Female and male silver-haired bats had assumed settling fuel load cost of $f_0 = 0.007$, male hoary bats had assumed settling fuel load cost of $f_0 = 0.006$, and female hoary bats had an assumed settling fuel load cost of $f_0 = 0.037$. Settling costs are a combination of the time and energy spent finding appropriate roosting and foraging locations upon arrival

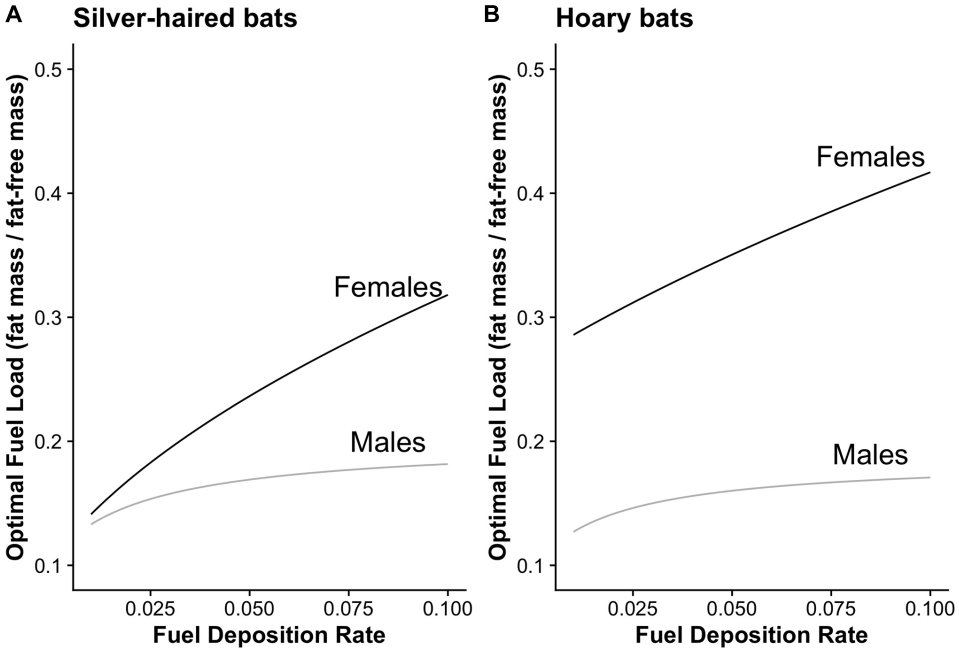

FIGURE 1 | Qualitative predictions of optimal fuel load across a range of fuel deposition rates for silver-haired bats and hoary bats using time and energy minimizing strategies as well as heterothermic and homeothermic thermoregulatory strategies. **(A)** The optimal fuel load for female heterothermic time minimizing silver-haired bats (black line) is predicted to be greater than for male heterothermic energy minimizing silver-haired bats (gray line). **(B)** Similarly, female homeothermic time minimizing hoary bats (black line) are predicted to have greater optimal fuel load than male heterothermic energy minimizing hoary bats (gray line), with the further prediction that the observed difference would be greater than the difference for silver-haired bats.

at the stopover site (i.e., time and energy spent before the start of active refueling).

To generate predictions of daily energy expenditure (MR_{field}) we summed energy costs associated with 2 h of flight and 22 h resting. In reality, settling cost and daily energy expenditure will change through time and space, however, qualitative predictions of fuel load are not overly sensitive to changes in daily energy expenditure parameters (Clerc and McGuire, 2021). For example if we assume 6 h of flight and 18 h resting, quantitative values will change but qualitative differences in predicted optimal departure fuel load between time and energy minimizers will remain relatively stable (Clerc and McGuire, 2021). We calculated metabolic rates using the mean body mass values for each group following the allometric scaling equations of Speakman and Thomas (2003) and used an oxyjoule equivalent of 20.09 ($J\ mL\ O_2{}^{-1}$) assuming a mixed fuel strategy (Speakman and Thomas, 2003; Lighton, 2018).

Field Predictions

We generated predictions of optimal fuel load as a function of stopover fuel deposition rate on a scale of 0.01 – 0.1 (where 0.1 represents a 10% daily increase in fuel mass) (**Figure 1**). For silver-haired bats we predicted females (time-minimizing heterotherm) would have greater fuel load than males (energy-minimizing heterotherm; **Figure 1A**). For hoary bats we predicted females (time-minimizing homeotherm) would have a greater fuel load than males (energy-minimizing heterotherm; **Figure 1B**). Furthermore, we predicted that the difference in fuel load between male and female hoary bats would be greater than the difference in silver-haired bats (**Figure 1**).

To achieve increased fuel loads we tested migration theory predictions that female hoary bats and female silver-haired bats conform to a foraging tactic of maximum net intake and thus would have an increased foraging proclivity and/or nutrient assimilation rate relative to males of both species. We predicted males of both species to conform to a foraging tactic of maximum efficiency.

Fuel Load

We measured fat mass (2016 and 2018 only) with quantitative magnetic resonance body composition analysis following the methods of McGuire and Guglielmo (2010). We supplemented our dataset with previously published (McGuire et al., 2013) fat and body mass data on 15 female and 15 male hoary bats captured at the same site during the same month. We express fuel load as a ratio of fat mass and lean mass. We considered lean mass to be fat-free mass and thus calculated fuel load as fat mass/(body mass – fat mass).

Foraging Assay: δ^{13}C Isotope Breath Signature

We measured the δ^{13}C isotope signature of the exhaled breath as an indicator of recent foraging activity (Voigt and Speakman, 2007). More enriched breath isotope signatures indicate a greater reliance on recently ingested prey for current metabolic demands, while depleted signatures indicate reliance on endogenous energy stores (Voigt et al., 2012). Therefore, this method can be used to determine which groups have greater foraging proclivity. We acquired breath samples following the methods of Voigt and Speakman (2007) and Voigt et al. (2012). If target species were captured and extracted from mist nets within 3 min (to avoid potential capture stress effects) we placed bats into a 500 ml glass container with a gas entry port connected to a tank of CO_2-free air, an exit valve, and a breath sample port. With the bat in the container, we pumped CO_2-free air through the container for 60 s to remove ambient CO_2 (a flow time empirically determined to evacuate containers of detectable CO_2 in the lab prior to field data collection). We then allowed exhaled breath to accumulate in the container for 240 s such that the CO_2 concentration reached 4,000 – 6,000 ppm, and collected the breath sample by penetrating the septum of two 13 mL exetainers (Labco Ltd, United Kingdom). Samples were stored at room temperature for up to 3 months prior to analysis on a CO_2 isotope analyzer run in batch mode (model CCIA-38-EP; LGR, Los Gatos, United States).

Nutrient Assimilation Rate Assay

To determine how rapidly bats oxidized recently ingested nutrients we fed fasted bats ^{13}C-labeled glycine (Glycine-1-13 C 99%, Sigma-Aldrich, United States) and measured the change in δ^{13}C of exhaled breath over a period of at least 45 min post-ingestion. We recorded the enrichment of exhaled breath at 10 min post-ingestion (arbitrarily selected common time point at which enrichment was rapidly increasing for all individuals) as an index of nutrient assimilation rate. Glycine is a non-essential (dispensable) amino acid that is preferentially metabolized by mammals, compared with essential (indispensable) amino acids, carbohydrates that can be stored as glycogen, or fat that can be stored in adipocytes (Welch et al., 2015). The rate at which a fasted bat assimilates ingested glycine can thus act as a proxy for the functional capacity of the gut under the assumption that there could be differences in the size of the gut (McGuire et al., 2013) or the density of transporters. Either increased gut surface area or greater density of transporters could lead to increased rate of appearance of oxidized glycine, allowing the rate of appearance to act as an index of gut functional capacity. Among Vespertilionid bats, sexual size dimorphism in body size (female > male) is common (Williams and Findley, 1979), and must be accounted for in statistical analyses (see below). Size dimorphism is especially pronounced for hoary bat females that may weigh ~30 – 50% more than males. Larger females presumably have larger digestive tracts, and therefore, all else being equal, should have increased rate of ^{13}C-labeled glycine appearance in the breath.

We randomly selected up to 6 individuals each night (prioritizing females due to male bias at our capture site) for the glycine assay [n = 64 silver-haired (2017 only) and n = 35 hoary bats (2017–2018)]. Bats were first fasted for at least 90 min to ensure that individuals voided recently ingested prey that may have interfered with their ability to digest and assimilate glycine (Buchler, 1975). We fed each individual 5 mg of ^{13}C-labeled glycine (Sigma Aldrich, United States) suspended in 200 μl of water. We placed up to 2 individuals in separate respirometry chambers and ran 0.60 L min^{-1} of CO_2-free air through the chambers and into the isotope analyzer. Flow-through mode acquired a semi-continuous reading of the δ^{13}C, using a flow multiplexer (Sable Systems, United States) to switch between each chamber every 30 s, such that we were able to document the rate of appearance of ^{13}C enriched breath. We provided mealworms to bats following all measurements and released all bats at the capture site.

Statistical Analysis

All analyses were completed with the program R (v 3.5.1; R Core Team, 2018). To test predictions of sex-based differences in fuel load, and that the difference would be greater in hoary bats than silver-haired bats, we used a linear model including sex, species, and the interaction between the two. We compared δ^{13}C isotope signature of exhaled breath with linear models including body mass, T_a (measured at sunset), and capture year as covariates. Because there is little overlap in body mass between male and female hoary bats, sex and body mass are confounded so we did not include mass in our initial model and then separately tested the effect of mass on ^{13}C isotope enrichment within each sex. We used a linear model to test for the effects of sex on assimilation rate, controlling for mass, capture date, and year. As above, we tested for the effect of mass within sexes. We used a backward stepwise model selection procedure for all linear models, sequentially removing non-significant terms until only significant terms remained. Because there is very little overlap in the body mass between sexes of hoary bats, sex and body mass are confounded so for both the digestive assay and glycine assay hoary bat analyses we first omitted mass in our models and then separated the sexes and tested mass effects on δ^{13}C isotope signature within males and females. We report Cohen's effect size values for all significant sex comparisons. All values are reported as mean ± s.e.m.

RESULTS

Fuel Load

Female silver-haired bats (n = 85) had a fuel load (0.11 ± 0.004) that was 34% greater ($F_{1,283}$ = 27.17, $p < 0.001$, Cohen's effect size d = 0.67) than male (n = 200) fuel load (0.082 ± 0.002) (**Figure 2A**). Similarly, female hoary bats (n = 45) had a fuel load (0.17 ± 0.008) that was 42% greater ($F_{1,72}$ = 21.97, $p < 0.001$, Cohen's effect size d = 1.12) than male (n = 29) fuel load (0.12 ± 0.007) (**Figure 2B**). The difference between female and male hoary bat fuel load was 79% greater than the difference between female and male silver-haired bats ($F_{1,355}$ = 7.96, p = 0.005).

Foraging Assay: δ^{13}C Isotope Breath Signature

Our results suggest that females and males of both study species forage during stopover (**Figure 3**). Female silver-haired bats (n = 132) had a mean δ^{13}C isotope signature of -27.09 ± 0.18 ‰ and male silver-haired bats (n = 67) had a mean isotope

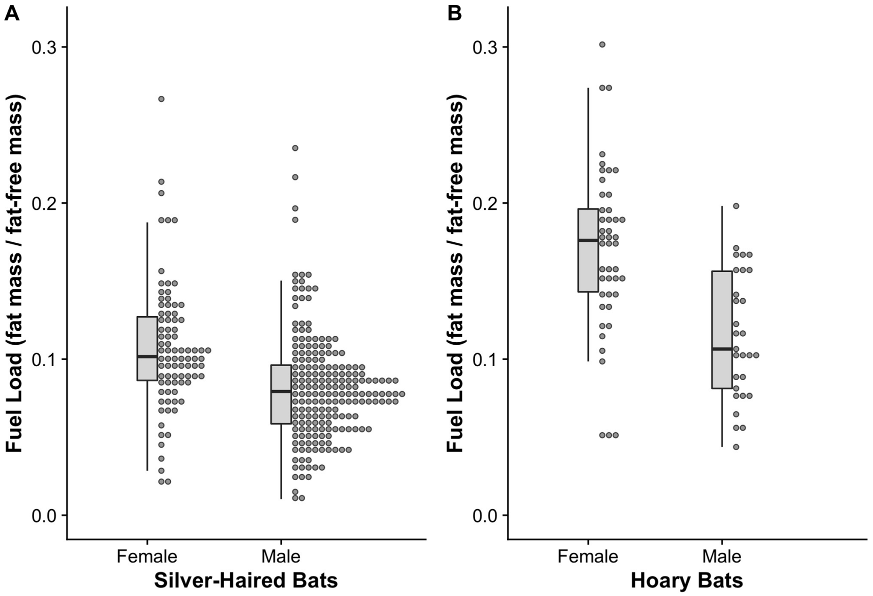

FIGURE 2 | Fuel loads of **(A)** spring migrating silver-haired bats (*Lasionycteris noctivagans*) and **(B)** hoary bats (*Lasiurus cinereus*). In both species, female fuel loads were greater than male fuel loads, consistent with optimal migration theory predictions based on females adopting a time-minimizing strategy and males adopting an energy minimizing strategy. Furthermore, the difference in fuel load is greater in hoary bats than silver-haired bats, consistent with predictions based on homeothermic female hoary bats and heterothermy among all others.

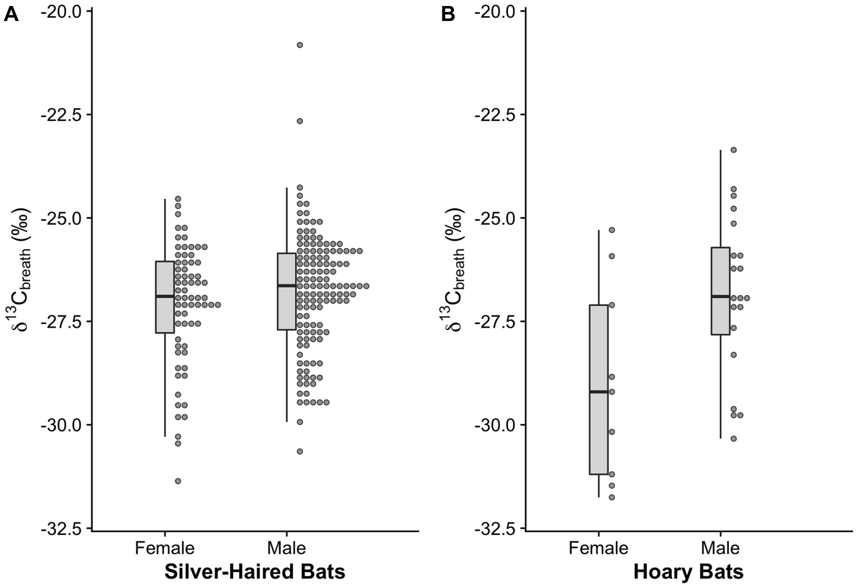

FIGURE 3 | ^{13}C enrichment in exhaled breath of spring migrating silver-haired and hoary bats. Less negative $\delta^{13}C$ values are associated with metabolism of exogenous nutrients (foraging) and more negative $\delta^{13}C$ values are associated with metabolism of endogenous fuel sources (relying on stored fat). Using relative ^{13}C enrichment as a proxy for foraging proclivity we find that **(A)** female and male silver-haired bats do not show differences in their proclivity to forage at stopover but that **(B)** female hoary bats are less likely to forage relative to male hoary bats.

signature of -26.79 ± 0.13 ‰ (**Figure 3A**). Male and female silver-haired bats did not differ in mean breath sample $\delta^{13}C$ enrichment ($F_{1,181} = 0.29$, $p = 0.588$), nor did sample ^{13}C enrichment vary across years ($F_{1,182} = 2.16$, $p = 0.143$). We detected a negative association of foraging with body mass ($F_{1,183} = 6.15$, $p = 0.014$), suggesting that individuals with lower mass were more likely to forage. We also detected a positive association of foraging with T_a ($F_{1,183} = 14.12$, $p < 0.001$), which aligns with prey availability increases as a function of T_a (Anthony and Kunz, 1977).

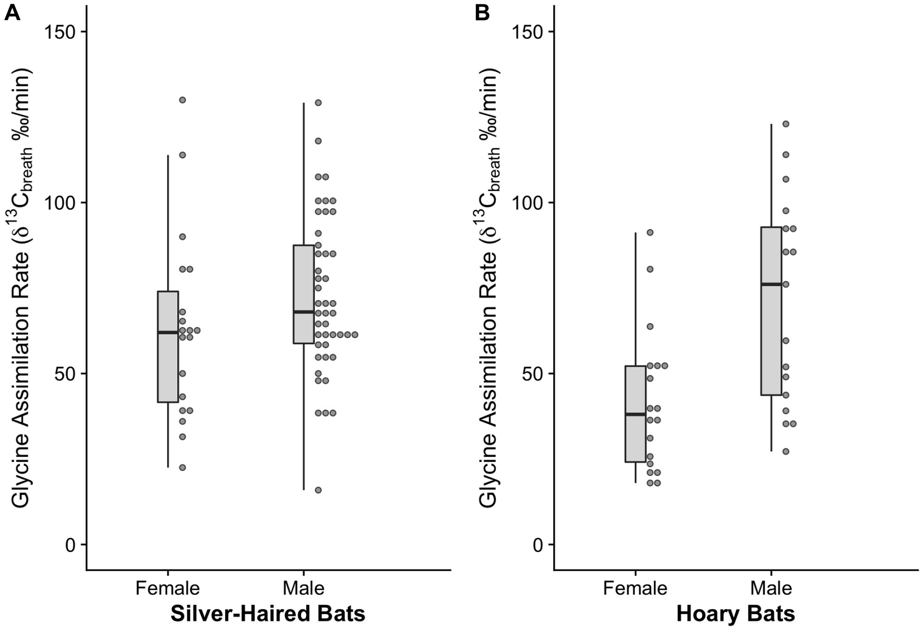

FIGURE 4 | ^{13}C enrichment 10 min post-ingestion of ^{13}C-labeled glycine (indicator of assimilation rate) in spring migrating silver-haired (*Lasionycteris noctivagans*) and hoary bats (*Lasiurus cinereus*). **(A)** Enrichment did not differ between female and male silver-haired bats, but **(B)** female hoary bats had depleted ^{13}C relative to males, suggesting reduced digestive capacity.

Female hoary bats (n = 9) had a more depleted δ^{13}C breath signature (−28.99 ± 0.81 ‰) than male hoary bats (n = 20; −26.84 ± 0.44 ‰; $F_{1,27}$ = 6.43, p = 0.017; Cohen's effect size, d = −1.02) (**Figure 3B**) signifying a greater reliance on endogenous fuel stores rather than recently ingested prey (Voigt et al., 2012; Baloun et al., 2020). Further, we did not find any effect of body mass on foraging proclivity for males ($F_{1,27}$ = 0.03, p = 0.865) or females ($F_{1,27}$ = 0.82, p = 0.396) suggesting a real effect of sex on foraging proclivity and that females are less reliant on recently ingested exogenous nutrients at our capture site than males. We did not detect any effects of year ($F_{1,25}$ = 0.23, p = 0.632), or T_a ($F_{1,25}$ = 0.99, p = 0.329) on hoary bat foraging proclivity.

Nutrient Assimilation Rate Assay

Glycine assimilation varied widely between the sexes of both species (**Figure 4**). There was no difference in mean glycine assimilation rate between male (72.68 ± 3.46 ‰/min; n = 45) and female (63.06 ± 6.29 ‰/min; n = 19) silver-haired bats ($F_{1,61}$ = 0.85, p = 0.360) (**Figure 4A**). Further, there was no effect of mass on glycine assimilation rate ($F_{1,60}$ = 0.01, p = 0.919). Glycine assimilation rate was positively related to capture date ($F_{1,62}$ = 18.42, p < 0.001), suggesting that either late migrants have greater gut functional capacity or that functional capacity increases over the course of migration.

Female hoary bats had a lower rate of ^{13}C-labeled glycine assimilation (41.79 ± 4.97 ‰/min, n = 18) relative to males (71.436 ± 7.45 ‰/min; n = 17; $F_{1,33}$ = 11.20, p = 0.002) and the difference was associated with a large effect size (Cohen's effect size, d = −1.13) (**Figure 4B**). There was no effect of body mass on the rate of apparent oxidation of ^{13}C-labeled glycine for males ($F_{1,15}$ = 0.001, p = 0.970) or females ($F_{1,16}$ = 0.07, p = 0.792). The rate of apparent oxidation of ^{13}C-labeled glycine was not affected by capture date ($F_{1,31}$ = 0.16, p = 0.691) or year ($F_{1,32}$ = 2.06, p = 0.161).

DISCUSSION

Our results are consistent with the migration theory predictions that females would have increased fuel loads relative to males and the difference between fuel loads between male and female hoary bats would be greater than the difference between male and female silver-haired bats. Fuel loads in both species qualitatively conformed with a time minimizing hypothesis for females and an energy minimizing hypothesis for males. Furthermore, relative differences in fuel load conform to the predicted effects of thermoregulatory expressions.

The prediction that female time minimizers maximize net energy intake and male energy minimizers maximize the foraging gain ratio at stopover was not supported. Despite increased fuel loads in females of both species, we found no evidence that females deposited more fuel than males at our capture site, either through increased foraging or increased digestive efficiency. We found no difference in foraging proclivity or nutrient assimilation in male and female silver-haired bats, and counter to our predictions found lower foraging intensity and nutrient assimilation in female hoary bats relative to males. The observed patterns of foraging and nutrient assimilation at our capture site are potentially explained by an inability to achieve positive energy balance (i.e., our study site may have been a low-quality stopover site), or that both sexes use the same foraging tactic (maximize net energy intake or maximize the foraging gain ratio). Explanations of the observed differential fuel loads then may include greater reliance on ephemeral blooms of high prey density, and/or reliance on carryover fuel from the pre-migratory period (see below).

Migration theory assumes that stopovers are used for refueling only when individuals can achieve positive energy balance. Unlike nocturnally migrating birds that can forage during the day and migrate at night, bats must manage both refueling and migratory flights during nocturnal periods, undoubtedly leading to foraging period time constraints. The challenges of gaining positive energy balance in a constrained time period are likely exacerbated when foraging quality is low. Torpor use may allow some migrants to overcome these constraints and build fuel at stopover, however, maintaining euthermia [i.e., as observed in pregnant hoary bats, Cryan and Wolf (2003)] may tip stopover energetics into negative balance across a large proportion of stopovers during migration. Increased stopover costs coupled with reduced foraging proclivity and a decrease in gut functional capacity suggest female hoary bats may not achieve positive energy balance on most occasions at our field site and may forgo foraging in favor of rapid departure. That females are also pregnant may further inhibit individual ability to achieve positive energy balance as increases in mass may make foraging flight energetically prohibitive unless prey availability and quality is high. Foraging proclivity, as indicated by ^{13}C enrichment in the breath, was low but variable in female hoary bats, suggesting opportunistic foraging may be a tactic used to mitigate rapid loss of endogenous fuel stores.

In our study, groups that presumably use torpor during migration exhibited a greater foraging proclivity than those that forgo torpor use. However, in the case of silver-haired bats there was no evidence that males and females used different foraging tactics. In conditions of low foraging quality and foraging time constraints, it is possible that maximizing net intake is the only foraging tactic that allows migrants to achieve positive energy balance. If this is the case, we might expect that rather than exhibiting a fixed foraging tactic throughout migration, individuals will adjust their foraging habits based on prey availability and time constraints. In conditions of high prey availability, males may switch tactic to maximize stopover foraging ratio, however, it is unclear if females would do the same or continue to maximize the net intake rate, as time-minimizers are predicted to. A logical extension of this research will be to sample across a wider range of habitat foraging qualities, to determine if males switch tactics to maximize efficiency when foraging quality is high, and if females maintain a net intake foraging tactic.

Females had greater fuel loads than males, but foraged less and did not have greater digestive efficiency. This begs the question of how females deposit or maintain greater fuel loads. One explanation is that females rely on ephemeral pulses of extreme prey abundance (Valdez and Cryan, 2009). Valdez and Cryan (2009) found that Geometrid and Noctuid moths make up the majority of hoary bat diets during spring migration at our study site. Females may only reach positive energy balance under foraging conditions where they encounter either emerging diapausal moths or migratory moths as they too move northward along the east slope of the Rocky Mountains (Valdez and Cryan, 2009; Krauel et al., 2015, 2017). When encountered, abundant moths would offer a more ephemeral, but substantial, foraging opportunity than might be encountered on average across the landscape. At our capture site we observed one such diapause emergence, where the ground surrounding the capture site was blanketed in presumably newly emerged Geometrid moths. In nearly 80 capture nights we have only encountered one such emergence, reinforcing the fact that bat prey is spatiotemporally ephemeral during spring migration.

Reliance on ephemeral prey pulses as the primary means of building large fuel loads is an inherently risky tactic. To consistently achieve increased fuel loads in this manner we would predict that gut functional capacity would be preemptively maintained at a high-performance level such that when pulses of high-density prey are encountered, individuals can take full advantage of their energic benefits. However, our nutrient assimilation assay suggested that females did not physiologically anticipate encounters with high density foraging opportunities. Females likely seek out high density pulses of prey, but there may be a trade-off in maintaining excess functional capacity in the gut (and the flight cost of carrying that extra load) that precludes maintaining high digestive efficiency throughout migration (Piersma and Gill, 1998; McGuire et al., 2013).

Another mechanism that would account for differential fuel loads is that females carry-over fuel from the overwintering period into spring migration. Unfortunately, little is known about the overwinter ecology of these species (Weller et al., 2016), so the feasibility of either increasing winter energy intake/reducing energy output relative to males or exhibiting pre-migratory hyperphagia is unknown. Despite gaps in the natural history, it is likely that overwintering behavior plays an important role in spring migration.

Theoretical models suggest silver-haired bats could achieve travel distances of >1500 km on fuel loads similar to those observed in the present study (McGuire et al., 2012). Thus, with maximum net migration distances of 1000 – 2000 km (Cryan, 2003; Cryan et al., 2014), hoary bat females may be able to arrive at summer grounds with minimal stopover refueling. However, if fuel stores become depleted, due to tortuous routing, or extended periods of inclement weather, and individuals can't locate high-density foraging opportunities, female hoary bats still have the ability to use torpor to save energy in emergency situations (Willis et al., 2006).

Our study highlights the strengths and limitations in generating predictions of stopover behavior under an optimal migration theory framework. Our target species appear to conform to the migration theory predictions of differential fuel load, however, we are unable to conclusively identify the mechanism by which differential fuel loads are achieved. In the case of migratory bat systems, we may need greater flexibility in the framework to incorporate previously unaccounted for mechanisms of fuel accumulation. Migration theory has been effectively used to generate predictions of stopover physiology and behavior, and with the extension of migration theory to account for heterothermic thermoregulatory strategies the framework can be used for an ever more diverse range of migrants. Empirical tests are critical to evaluate model predictions and determine how well the models fit observed patterns of migration. Through an iterative process of modeling and empirical testing we can refine migration theory to develop a comprehensive understanding of the ecological patterns of migration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All procedures were approved by the Texas Tech University Institutional Animal Care and Use Committee (protocols 16013-04 and 19043-05) and permits from the New Mexico Department of Game and Fish (permit 3424).

AUTHOR CONTRIBUTIONS

JC and LM contributed to conception and design of the study and performed the statistical analysis. JC, ER, and LM contributed to data collection. JC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Hyper- and Hypo-Osmoregulatory Performance of Atlantic Salmon (*Salmo salar*) Smolts Infected With *Pomphorhynchus tereticollis* (Acanthocephala)

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Migratory species must cope with different parasite communities in different environments, but little is known about the ecophysiological effects of parasites on migratory performance. Some species/strains of acanthocephalan parasites in the genus *Pomphorhynchus* use anadromous salmonids as preferred definitive hosts, perforating the intestines, destroying mucosa and inducing inflammation–all of which might affect osmoregulatory function during transition between freshwater and marine environments. We used genetic barcoding to identify acanthocephalans in the intestines of wild Irish Atlantic salmon (*Salmo salar* L.) smolts as being the recently taxonomically resurrected species *Pomphorhynchus tereticollis*. We then investigated whether natural infection intensities of this parasite were associated with reduced osmoregulatory performance, as measured by plasma chloride concentrations, or potentially elevated stress, as measured by blood glucose, of hosts in freshwater or saltwater environments (24 or 72 h in ∼26PPT salt water, reflecting salinities of coastal waters through which smolts migrate). Although infection prevalence was high amongst sampled smolts, no associations were found within or across treatment groups between parasite abundance and plasma chloride concentrations or blood glucose levels. We found no intestinal perforations that would indicate *P. tereticollis* had recently vacated the intestines of smolts in either of the saltwater groups. Exploratory sampling in the 2 years preceding the experiment indicated that parasite prevalence and abundance are consistently high and comparable to the experimental individuals. Collectively, these results indicate that naturally occurring abundances of *P. tereticollis* do not reduce osmoregulatory function or affect blood glucose content in fresh water or within 72 h of entering coastal waters, although delayed pathologies affecting marine survival may occur. Future consideration of ecophysiological interactions between anadromous fish hosts and their parasites across different osmotic environments should provide general insights into coevolution between migratory hosts and their parasites.

Keywords: parasite, stress, osmoregulation, anadromy, salmonid, *Pomphorhynchus tereticollis*

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INTRODUCTION

Migration has evolved in a diverse array of animal taxa as a strategy for coping with, or exploiting, spatiotemporal environmental variation (Dingle, 2014). Physiological processes (e.g., metabolic and neuroendocrine pathways) drive how animals translate environmental cues into movement decisions (Goossens et al., 2020) and also mediate fitness costs of these decisions (Alerstam et al., 2003; Brownscombe et al., 2017) and consequent life-history trade-offs (Ricklefs and Wikelski, 2002). While the performance of migratory aquatic species has been clearly linked to a range of abiotic drivers such as temperature (e.g., Crossin et al., 2008; Gilbert and Tierney, 2018), oxygen limitation (e.g., Rosa and Seibel, 2010), water flow (e.g., Swanson et al., 2004), water chemistry (e.g., Borges et al., 2019), and pollution (e.g., Seewagen, 2020), the role of biotic factors in the ecophysiology of animal migration is arguably less well studied. In particular, parasites and pathogens have received relatively little attention, but are critical components of any ecosystem and are likely to exert a range of physiologically mediated influences on migration decisions and fitness outcomes (Piersma, 1997; Altizer et al., 2000; Gylfe et al., 2000; Norris and Evans, 2000; Møller and Szép, 2011).

Salmonid fishes (Family Salmonidae) are a group of broad ecological, economic, and cultural importance that exhibit a diversity of migratory strategies, ranging from residency, to potamodromy (migrations within freshwater) to anadromy (migration to sea for growth followed by return to freshwater for spawning). Their complex life cycles and amenability to experimental work and tracking studies has made salmonids the focus of much research in ecophysiology (see reviews by: McCormick et al., 1998; Poole et al., 2003; Hinch et al., 2005; Cooke et al., 2008, 2012; McCormick et al., 2009; Groot, 2010; Björnsson et al., 2011; Eliason and Farrell, 2016). The life histories of anadromous species such as Atlantic salmon (*Salmo salar* L.) necessitate the regulation of internal osmotic balance in hypoosmotic (freshwater) and hyperosmotic (saltwater) environments. In fresh water, osmoregulation requires excess water, which is passively absorbed through osmosis across the gills and skin, to be excreted as dilute urine (Genz et al., 2011). In contrast, osmoregulation in a marine environment requires salmon to continuously drink salt water and actively uptake H_20 through the intestinal epithelium into the body, while limiting intestinal absorption of ions in order to mitigate diffusive water losses through the gills and skin (Grosell, 2007; Whittamore, 2012).

The initial period of acclimation to the marine environment necessitates significant changes to the internal physiology of salmon smolts (Stefansson et al., 2012) and provides an acute physiological challenge (Handeland et al., 2014). Plasma cortisol levels rise during the parr-smolt transformation, and this natural stress response is thought to benefit smolts by mobilizing energy reserves and increasing saltwater tolerance (Bisbal and Specker, 1991; Strand and Finstad, 2007). However, further sources of acute or chronic stress can greatly impair osmoregulatory ability in salmonid smolts (Redding and Schreck, 1983; Iversen et al., 1998) as observed with infestations of ectoparasitic sea lice (*Lepeophtheirus salmonis*) (Poole et al., 2000) or *Gyrodactylus salaris* (Bakke and Harris, 1998). Parasite-induced damage to organs involved in osmoregulation such as the skin, gills or intestines can also directly impact the ability of salmonids to maintain osmotic homeostasis in salt water, leading to disruption of physiological processes, elevated stress, and ultimately mortality (Dawson et al., 1998; Wells et al., 2006; Finstad et al., 2012; Hvas et al., 2017). Through these pathological effects, increased sea lice infestations associated with fish farming are regarded as a significant factor contributing to declines in the marine survival of many Atlantic salmon and sea trout (anadromous *Salmo trutta* L.) stocks in recent decades (Poole et al., 2007; Krkošek et al., 2013; Thorstad et al., 2015; Shephard and Gargan, 2017).

Pomphorhynchus tereticollis is an acanthocephalan endoparasite of various freshwater and brackish fishes with a complex (heteroxenous) life cycle that requires infection of both intermediate and definitive host species. Previously regarded as a synonym for the phenotypically similar *Pomphorhynchus laevis*, recent genetic characterisation has led to the resurrection of *P. tereticollis* as a distinct species within the (former) *P. laevis* complex (Špakulová et al., 2011). Based on morphological and molecular examinations of new and archived material, recent studies provide convincing evidence that *P. tereticollis* is the only *Pomphorhynchus* species present in Ireland and Britain, and that all previous literature pertaining to *P. laevis* in this region represents misidentifications of *P. tereticollis* (Perrot-Minnot et al., 2018; Andreou et al., 2020; Tierney et al., 2020). To avoid confusion, we use the term *P. laevis s.l.* (*sensu lato*) when referring to older studies in which *P. tereticollis* may have been misidentified as *P. laevis*, or to more recent studies in which the taxonomy was uncertain. Such studies have shown that *P. laevis s.l.* infection rates of Irish brown trout peak in spring, coinciding with the annual smolt run (Molloy et al., 1995), and that parasite abundance and prevalence are highest in smolt-aged Atlantic salmon (i.e., 2+) (Fitzgerald and Mulcahy, 1983). Pippy (1969) found that the incidence of *P. laevis s.l.* in Atlantic salmon smolts in Ireland was 25 times higher than in Scotland, England and Wales. Thus anadromous salmonids in Ireland have a particularly high chance of entering salt water while infected by these acanthocephalan parasites.

Pomphorhynchus tereticollis uses gammarid species as intermediate hosts and trophic transmission to a definitive host requires the consumption of an infected gammarid by a suitable fish species (Perrot-Minnot et al., 2020). Upon consumption by a salmonid, *P. tereticollis* use their hooked proboscis to pierce all layers of the intestinal wall and anchor themselves in place. This process creates a perforation leading from the interior to the exterior of the intestinal wall, destroying intestinal mucosa, causing a localized inflammatory response and potentially altering the physiological performance of the intestine in controlling transepithelial ion transport (Wanstall et al., 1986, 1988; Dezfuli et al., 2002b, 2008). Previous studies have concluded that, despite causing such intestinal damage, infection with these acanthocephalans does not significantly reduce growth rates in salmonids and does not directly cause mortality of the host (Hine and Kennedy, 1974; Wanstall, 1984;

Wanstall et al., 1986). However, these studies have focused on the impact of acanthocephalan infection on salmonids in fresh water where osmoregulation does not require equivalent control of transepithelial ion transport or active H_20 uptake through the intestinal wall, as is necessary in saltwater environments.

Exposure to sub-optimal conditions or stressors in fresh water has been shown to reduce osmoregulatory performance and increase the susceptibility of salmonids to parasitic infection and associated mortality in the marine environment (Finstad et al., 2012, 2007). In the present study, we first used genetic barcoding techniques to confirm that Atlantic salmon smolts in our Irish study system are infected by *P. tereticollis*. We then investigated whether natural infection intensities of *P. tereticollis* in wild-caught smolts affect their osmoregulatory performance in saltwater or freshwater environments, and whether such osmoregulatory impacts might also be associated with potentially increased stress. We hypothesized that perforations made by *P. tereticollis* in the intestinal wall would allow uncontrolled ingress of water into the peritoneum, while parasite-induced damage to mucosa, and the associated inflammatory response, would further reduce the ability of salmonids to control ion uptake or water absorption through the intestines. Through these processes, *P. tereticollis* infection was predicted to compromise the osmoregulatory performance of Atlantic salmon in salt water (i.e., hypo-osmoregulation), leading to elevated stress and increased ion concentrations in the blood within marine environments. While we regarded a strong reduction in hyper-osmoregulatory performance as less likely, we predicted that pathological effects of *P. tereticollis* infection (including reduced control of transepithelial ion transport) could also lead to elevated stress and, potentially, reduced blood ion concentrations in freshwater environments.

We characterized *P. tereticollis* infection prevalences and intensities in wild smolts captured from the Burrishoole catchment, Co. Mayo, over a 3-year period and investigated whether infection patterns were associated with smolt size, sex or condition. Smolts captured on the third year were held in fresh (n = 66) or salt (n = 132) water prior to sampling and blood samples were extracted shortly after euthanasia. We then tested whether higher parasite abundance (higher numbers of *P. tereticollis* per individual) was associated with reduced osmoregulatory performance of smolts within each osmotic environment (fresh or salt water) as indicated by plasma chloride concentrations, which provide a direct measure of internal osmotic balance (Strand and Finstad, 2007; McCormick, 2012; Archer et al., 2019). We also measured blood glucose levels as one potential indicator of physiological stress (McGeer et al., 1991) that might accompany impaired osmoregulatory performance in fresh or salt water environments.

MATERIALS AND METHODS

Exploratory Sampling

Wild Atlantic salmon smolts were captured for stock assessment purposes at the tidal limit of the Burrishoole river system (NW Ireland; 53° 55′ 13 N, 9° 35′03 W) in May 2016 (n = 136) and May 2017 (n = 39). The Burrishoole system is comprised of over 45 km of small rivers and streams that link two main freshwater lakes, Bunaveela Lough (46 ha) and Lough Feeagh (410 ha), and ultimately flow into Lough Furnace (141 ha), a brackish, partially tidal lake opening into Clew Bay (Matthews et al., 1997; Whelan et al., 1998). The majority (∼89%) of salmon from the Burrishoole catchment smoltify and migrate to sea as 2 year old fish (Fealy et al., 2014; de Eyto et al., 2016). Captured smolts were dissected and their digestive tracts inspected for the presence of acanthocephalan parasites. Attached and unattached acanthocephalans in each smolt were recorded and stored in ethanol for subsequent DNA barcoding work. Smolt weight (to 0.1 g) and fork length (to 1 mm) were recorded before dissection and sex was determined by inspection of gonads during removal of the digestive tract. Chi-square and Mann-Whitney U tests were used to investigate whether there was a significant relationship between acanthocephalan prevalence or infection intensity, respectively, and sex amongst the smolts sampled in 2016, 2017 or amongst the experimental 2018 samples.

Experimental Setup

On two occasions during 2018, emigrating wild smolts (mean fork length = 138.9 mm, SD = 9.5 mm, range = 121–168 mm) were captured at the Salmon Leap fish trap located at the confluence between the freshwater component of the Burrishoole river system and the saline environment of Lough Furnace and Clew Bay (de Eyto et al., 2020). On each occasion, captured smolts were transported <100 m to an indoor Marine Institute research facility where they were transferred in an *ad hoc* fashion to evenly populate four 500 L aerated experimental tanks. On the first capture occasion (02 May), 66 smolts were distributed evenly amongst four tanks that had each been filled with 300 L of fresh water (i.e., 16–17 smolts per tank). After 24 h all 66 smolts were terminally sampled (see next section), at which point the experiment finished for this freshwater treatment group (24FW) and the four tanks were emptied of water.

On the second capture occasion (05 May), 132 smolts were distributed evenly amongst the same four tanks, each now pre-filled again with 50 L of fresh water (i.e., 33 smolts per tank). During the 2 h after the 132 smolts were transferred, 300 L of locally sourced sea water were gradually added to each tank, raising the salinity in each tank to 26.1–26.3 PPT at a rate that reflects the natural salinity increase experienced by wild smolts moving from the Burrishoole system to coastal waters. Twenty-four hours after the salinity had reached this peak, 66 smolts (16–17 smolts per tank) were terminally sampled and this group then comprised the 24 h in saltwater (24SW) treatment group. The remaining 66 smolts were then terminally sampled 48 h later, i.e., after a total of 72 h in saltwater (72SW). Water temperatures ranged between 8.4 and 13.9°C and dissolved oxygen was maintained at >8.5 mg/L during all phases of the experiment. The tanks were covered throughout the experiment in order to reduce exposure to potential external sources of stress.

Experimental Sampling Procedure

At each sampling time (i.e., 24FW, 24SW, and 72SW), dip nets were used to transfer 16–17 smolts from each of the four tanks into a pH buffered solution of tricaine methanesulfonate (450 mg L^{-1}) while minimizing disturbance to the remaining smolts. Smolts were monitored until opercular movement ceased and death was confirmed by severing the spinal cord with a scalpel (completing the killing of the animal in accordance with Annex IV of EU Directive 2010/63/EU and Irish Statutory Instrument 5432 of 2012). Blood samples were extracted from the caudal vein (along midline just posterior of the anal fin) with 1 ml 21G lithium-heparinised syringes (containing ∼6 USP units of lithium-heparin and providing ∼15 USP units per ml of blood) immediately after cervical dislocation and transferred to 1 ml Eppendorf© tubes which were stored on ice. Mean duration between dip netting and blood sampling was 9 min and 24 s (SD = 261 s, max = 1110 s, min = 121 s).

Sample Processing

A commercially available meter (FreeStyle Lite: Abbott) was used to take a single measurement of the blood glucose level (mmol/L) of each smolt within 1 min of sacrifice, requiring one drop of blood from the needle of each syringe. This meter has been shown to accurately measure glucose levels in teleosts (Eames et al., 2010). Each smolt was then weighed (to 0.1 g), measured (fork length "FL" to 1 mm), and a ∼2 mm^2 clip of caudal tissue was stored in ethanol for genetic sex determination. The condition factor (Fulton's *K*) for each smolt was then calculated by the following formula (Ricker, 1975):

$$K = \frac{W}{FL^3} \times 100,$$

where *K* is condition factor, W is smolt weight (g) and FL is fork length (cm).

Carcasses were placed in individual sealable plastic bags and stored on ice until dissection. All smolts were dissected within 8 h of mortality. An incision was made along the midventral line and the alimentary tract was removed after severing its junctures with the anus and the esophagus. The phenotypic sex of each smolt was determined by visual inspection of the gonads and in any case where the designation was uncertain genetic methods were used to verify sex (as per Finlay et al. (2020)).

Once removed from the body, the alimentary tract of each smolt was temporarily filled with water and pinched at each end to create water tight seals. The oesophageal end was then compressed to pressurize the internal water and the external wall was closely inspected for "pinprick" leaks that would indicate the presence of unplugged perforations left by previously attached acanthocephalans. The alimentary tract was then opened by mesial incision with a fine-point scissors and divided into four sections; (1) stomach (esophagus to pyloric caeca), (2) anterior intestine (33% of intestinal length from post-pyloric caeca to rectum); (3) intermediate intestine (middle 33% of intestine), and (4) posterior intestine (last 33% of intestine ending at anus). Each section was examined for the presence of parasites and the number of attached and unattached acanthocephalans in each section was recorded. On each sampling date, 30 acanthocephalans were examined under a microscope within 15 min of opening the digestive tract and their status as "alive" or "dead" was determined based on the presence or absence of observable movement in response to physical stimulus. A subset of acanthocephalans (*n* = 264) were also weighed in groups of 2–32 individuals (each group collected from a single smolt) and mean individual parasite weight per group and in total were calculated. Parasite abundance was measured as the number of acanthocephalans in an individual host, regardless of whether the host was infected or not. We calculated the prevalence of infection as the percentage of smolts containing acanthocephalans and infection intensity as the number of acanthocephalans in infected individuals.

Plasma was separated from all blood samples within 4 h of extraction by spinning in a centrifuge (ALC PK 421) at 3000 rpm for 10 min. Where possible, 0.07 ml of plasma was extracted from each sample (12 samples provided less than 0.07 ml of plasma) with an adjustable micropipette (Nichipet Ex) and stored in a 0.5 ml tube at −20°C for chloride analysis. Plasma chloride was measured by coulometric titration using a Jenway PCLM3 chloride meter (reproducibility ±1% or ±1 mmol/l for 100 μl sample at 100 mmol/l). Where plasma quantities were sufficient (98% of samples), chloride samples were tested in duplicate, and triplicates were run for any samples showing a difference greater than three units between the first two replicates. All blood assays were conducted within 3 weeks of freezing plasma.

Molecular Species Identification

Twenty-two of the attached acanthocephalan parasites, representing two randomly selected acanthocephalans from each of eleven randomly selected experimental smolts, were selected for molecular confirmation of species identity. DNA extraction was performed on entire specimens using the DNeasy® Blood and Tissue Kit (Qiagen), following instructions provided in the kit handbook. A 558 bp region of the mitochondrial DNA COI gene was amplified using the PT/PL-COI primers described in Tierney et al. (2020). PCR was performed in a 20 μl total volume consisting of 10 μl of 2× Plain Combi PP Master Mix (Top-Bio), 1 μM each of forward and reverse primer and 10–50 ng of DNA. PCR cycling conditions were as follows; an initial denaturation step of 3 min at 95°C was followed by 35 cycles of 94°C for 30 s 51°C for 30 s and 72°C for 60 s, with final extension step of 72°C for 5 min. Electrophoresis of PCR products was performed on 1% agarose and products were excised and purified using a QIAquick™ Gel Extraction Kit (QIAGEN). Sequencing was performed from both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were purified using the EDTA-ethanol precipitation method described in the sequencing kit handbook and were run on an ABI3500XL DNA analyzer.

Ethics Statement

We adhered to the ASAB/ABS Guidelines for the Use of Animals in Research throughout this project. All actions relating to the capture and sampling of smolts as well as the manipulation of environmental salinity were carried out in accordance with S.I. No. 123/2014 Animal Health and Welfare (operations and procedures) Regulations 2014 and with approval of the Marine Institute animal welfare committee (MI Establishment Authorisation No: AE19121) and the Health Professionals

Regulatory Authority (HPRA Classification Request Number: 066). Procedures for euthanasia were appropriate for salmonids (Popovic et al., 2012). Sampling was carried out by personnel with appropriate training and Individual Authorisations under Scientific Animal Protection Legislation (HPRA).

Statistical Analyses

We conducted all analyses using the statistical computing software R v3.6.1 (R Core Team, 2019). We specified separate generalized least squares models (GLS) using the *gls* function in the *nlme* package (Pinheiro et al., 2019) to investigate the extent to which variation in blood parameters (blood glucose and plasma chloride) was associated with variation in two continuous (acanthocephalan count, i.e., parasite abundance, and smolt condition factor, measured as Fulton's K) and four categorical (treatment group, sex, operator, and tank) explanatory variables. Treatment group had three levels (24FW, 24SW, and 72SW) corresponding with the three sampling dates. Operator, with two levels designating the two operators who performed the blood sampling, was included to control for potential variation in response variables resulting from differences in sampling technique among personnel. Tank had four levels reflecting the four experimental water tanks and sex had two levels, male and female. GLS models were used in order to account for differences in the variance of each of the three response variables observed amongst the three treatment groups, i.e., to control for heteroscedasticity. Initial models included an interaction between parasite abundance and treatment group in order to test whether any effect of acanthocephalan infection on chloride or blood glucose depended on treatment group (i.e., whether parasite effects on these physiological parameters varied between hyper and hypo-osmotic environments, and whether effects increased with time spent in hyper-osmotic environments). Models excluding this interaction were then run in order to test whether parasite abundance was associated with variation in these blood parameters independently of treatment group. AIC values were used to compare models including and excluding the interaction between parasite abundance and treatment group.

We used the *glmmTMB* function (Brooks et al., 2017) to specify generalized linear models (GLMs) to explore the degree to which the variation in infection prevalence and individual infection intensity was associated with variation in the condition factor (Fulton's K), length, weight and sex of smolts sampled in 2016, 2017, and 2018 (n = 312, the subset for which sex and size were both recorded). The individual infection intensity model included only smolts that were infected (n = 257). We used negative binomial models with log link functions to investigate individual infection intensity, in order to account for residual overdispersion in the data, and binomial models with logit link functions to investigate infection prevalence. Due to high collinearity between fork length and weight (R^2 = 0.91), two models were specified for each response variable (i.e., infection prevalence and intensity), each including either fork length or weight, and AIC values were used to compare both models. As both the infection prevalence and infection intensity models containing fork length yielded marginally lower AIC values than the models containing weight (−0.4 and −0.6, respectively), only results from the models with fork-length are presented.

Prior to model fitting, collinearity between all continuous explanatory variables in each model was explored by Pearson's R with the *cor.test* function in the *stats* package and associations between continuous and categorical explanatory variables were examined visually. Variance inflation factors (VIFs) were calculated for all fixed effects in each GLM with the *check_collinearity* function in the *performance* package in R (Lüdecke et al., 2019). We tested for heteroscedasticity and violations of linearity amongst residuals from the GLMs by plotting fitted values against simulated (scaled) residuals with the *DHARMa* package (Hartig, 2019). We tested for temporal autocorrelation with the *acf* function in the *stats* package. The *qqnorm* and *plot* functions were used to investigate residual distributions from GLS models. Chi-square tests were used to investigate whether there were significant differences in acanthocephalan prevalence or abundance amongst experimental treatment groups or amongst tanks within each treatment group.

RESULTS

Molecular Identification of Acanthocephalan Parasite Species

All 22 acanthocephalan specimens examined were identified unambiguously as *P. tereticollis*, with 97–100% sequence match to voucher specimens. Sequence match with *P. laevis* was less than 90% in all cases.

Parasite Prevalence, Infection Intensity and Locations Within the Alimentary Tract

Acanthocephalan infection prevalences amongst smolts sampled in 2016, 2017, and 2018 were 74.2, 64.1, and 66.2%, respectively. Mean infection intensities amongst the 2016, 2017, and 2018 samples, respectively were 9.23, 7.28, and 6.9 acanthocephalans per infected smolt. Infection prevalence amongst male and female smolts, respectively was 76.2 and 75% in 2016 (χ^2 = 0.77, p = 0.68), 93.8 and 40.9% in 2017 (χ^2 = 11.82, p = 0.003), and 71.1 and 63.6% in 2018 (χ^2 = 0.84, p = 0.358). Mean infection intensity per infected smolt amongst males and females, respectively was 12.8 and 4.9 in 2016 (Mann-Whitney U test: W = 1171, p = 0.044), 7.9 and 7.0 in 2017 (W = 53.5, p = 0.416) and 6.0 and 7.6 in 2018 (W = 2063, p = 0.942). Ten of the 198 smolts sampled in 2018 contained unattached acanthocephalans with nine of these smolts also containing attached acanthocephalans. A total of 899 attached and 15 unattached acanthocephalans were recorded amongst the 2018 experimental samples and the number of attached worms per smolt ranged from 1 to 42 (**Figure 1**).

Almost all (93.64%) attached acanthocephalans were in the intermediate (central 33%) section of the intestine while 3.11 and 3.25% were located in the anterior and posterior sections, respectively. No attached or unattached acanthocephalans were found in the esophagus, stomach or pyloric caeca. A few (n = 7) acanthocephalans were attached to the muscle along the

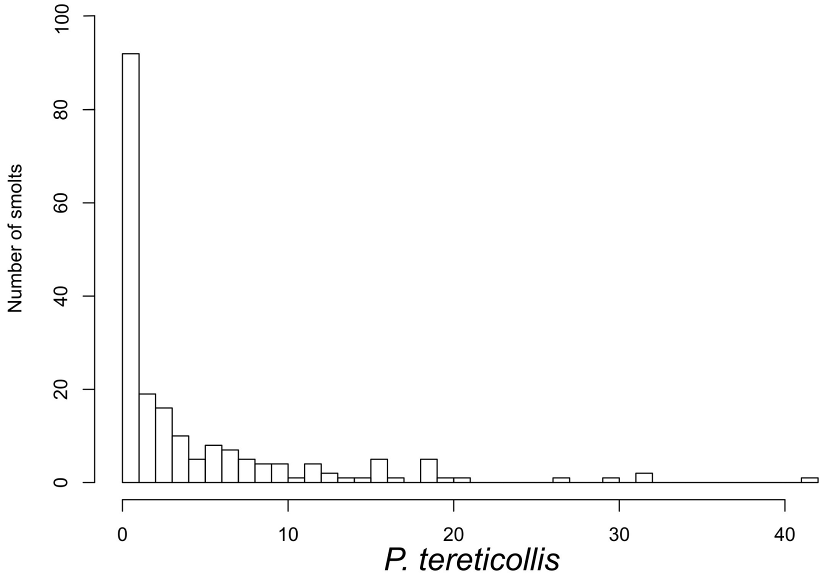

FIGURE 1 | Histogram of *P. tereticollis* abundance for all smolts used in experiment ($n = 198$).

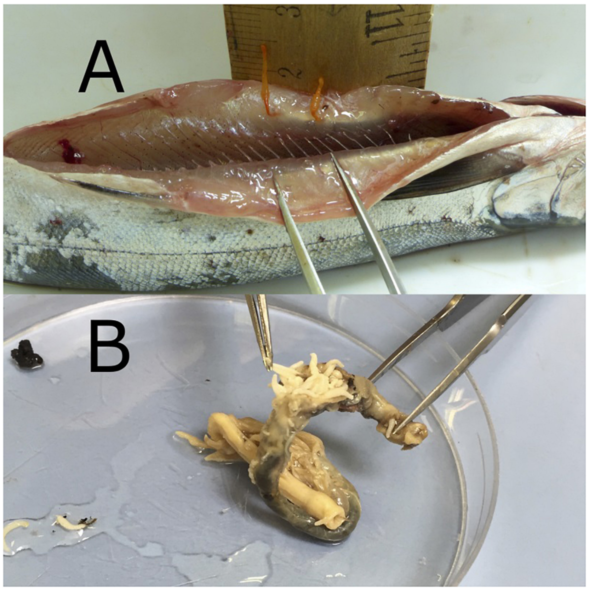

FIGURE 2 | (A) Photograph of *P. tereticollis* outside the alimentary tract of salmon smolt and attached to the peritoneum and muscle wall. **(B)** Salmon smolt intestine, heavily infected by *P. tereticollis*.

inside wall of the peritoneal cavity, having presumably passed completely through the wall of the digestive tract (**Figure 2A**). The mean weight of individual attached worms was 3.1 mg (SD of mean individual weight per smolt = 1.5 mg, max = 7 mg, min = 1.5 mg). All worms from all treatment groups that were observed under a microscope directly after removal from the intestines were found to be alive. Infection prevalence in the 24FW, 24SW, and 72SW treatment groups were 63.1, 56.9, and 77.6%, respectively ($\chi^2 = 6.7$, $p = 0.036$). Mean acanthocephalan counts per smolt in the 24FW, 24SW, and 72SW treatment groups were 4.29, 3.48, and 5.88, respectively ($\chi^2 = 40.5$, $p = 0.769$). Acanthocephalan prevalence did not differ significantly amongst the four tanks in the 24FW ($\chi^2 = 4.9$, $p = 0.183$), 24SW ($\chi^2 = 6.7$, $p = 0.084$), or 72SW ($\chi^2 = 0.8$, $p = 0.841$) treatment groups. Acanthocephalan count did not differ significantly amongst the four tanks in the 24FW ($\chi^2 = 53.2$, $p = 0.507$), 24SW ($\chi^2 = 43.9$, $p = 0.390$), or 72SW ($\chi^2 = 49.3$, $p = 0.657$) treatment groups.

No evidence of damage to intestine walls (i.e., pinprick leaks or visible perforations) from recently expelled acanthocephalans was observed in sampled smolts. Additionally, no leakage was observed through intestinal perforations that were plugged by the probosces of acanthocephalans. No mortality of smolts occurred in any treatment group prior to sampling. The binomial model revealed a significant positive association between infection prevalence and Fulton's K ($p = 0.012$) (**Figure 3A**) but no evidence of significant associations with sex or fork length (**Table 1**). The negative binomial model (**Table 2**) revealed a significant association between individual infection intensity and sex, with infected males having higher infection intensities than infected females ($p = 0.043$). This model also revealed a non-significant positive association between Fulton's K and infection intensity ($p = 0.091$) (**Figure 3B**).

Blood Parameters

The mean blood parameters in each treatment group fell within reported ranges for Atlantic salmon (**Table 3**; Bowers et al., 2000; Finstad et al., 2012; Kolarevic et al., 2014). Our initial chloride model revealed no significant interaction between acanthocephalan count and treatment group ($p = 0.447$). When this interaction was excluded, the model AIC value decreased

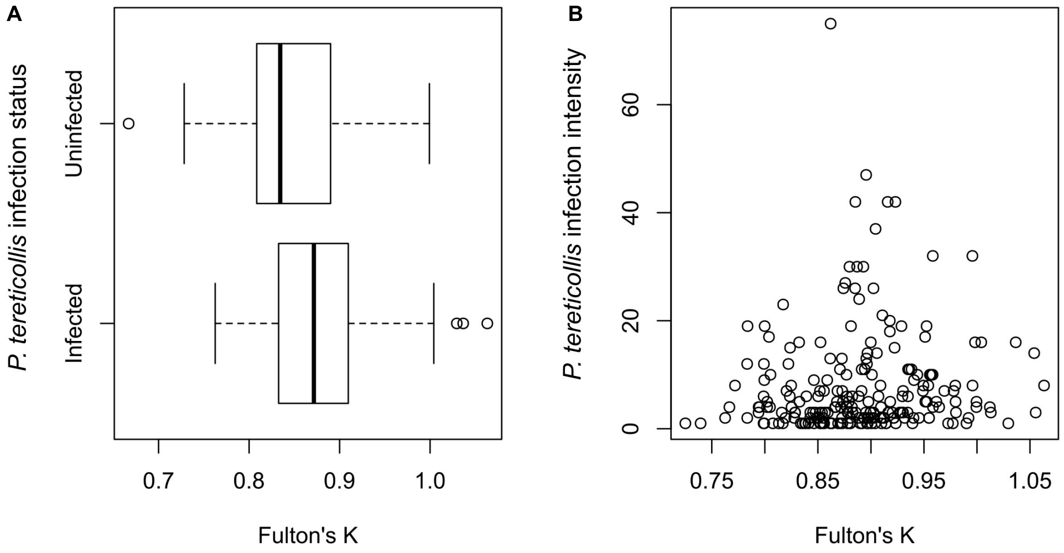

FIGURE 3 | (A) Boxplot of infection status (i.e., whether individual smolts contain *P. tereticollis* or not) vs. Fulton's K (condition factor) ($n = 312$) and **(B)** scatterplot of individual infection intensity (i.e., the number of *P. tereticollis* per infected smolt) vs. Fulton's K ($n = 218$). Data include smolts sampled in 2016, 2017, and 2018.

TABLE 1 | Parameter estimates for the binomial prevalence GLM where individual infection status (i.e., infected or uninfected) is the binary response variable.

	Estimate	Std. Error	*z* value	*p* value
(Intercept)	−3.212	2.793	−1.150	0.250
Fultons' K	5.339	2.113	2.527	0.012
Fork Length	−0.054	0.126	−0.426	0.670
Sex: Male	0.252	0.255	0.990	0.322

The intercept corresponds to the estimates (on the logit scale) for females.

TABLE 2 | Parameter estimates for the negative binomial GLM where individual infection intensity (i.e., the number of *P. tereticollis* per infected smolt) is the response variable.

	Estimate	Std. Error	*z* value	*p* value
(Intercept)	−1.338	1.497	−0.893	0.372
Fultons' K	2.030	1.201	1.690	0.091
Fork Length	0.104	0.063	1.640	0.101
Sex: Male	0.276	0.137	2.023	0.043

The intercept corresponds to the estimates (on the log scale) for females.

TABLE 3 | Mean and standard deviations for each blood parameter and physical measurements for each treatment group.

Treatment Group	24FW	24SW	72SW
Chloride	103.9 ± 10.1	117.7 ± 3.9	119.3 ± 4.8
Glucose	6.7 ± 2.6	4.0 ± 0.9	3.3 ± 0.7
Fork length (mm)	139.9 ± 9.1	138.1 ± 7.9	138.7 ± 8.5
Weight (g)	24.5 ± 5.1	22.8 ± 4.1	23.1 ± 4.7
Condition factor (K)	0.884 ± 0.063	0.858 ± 0.054	0.855 ± 0.058

by ∼7 and the main effect of acanthocephalan count was non-significant ($p = 0.26$) (**Figure 4**). The model without an interaction revealed significantly higher plasma chloride concentrations in the 24SW and 72SW treatment groups relative to the 24FW group (**Tables 3**, **4**). This model also revealed a significant negative relationship between Fulton's K and plasma chloride (**Table 4**). However, acanthocephalan count was not associated with variation in plasma chloride.

Our initial glucose model revealed no significant interaction between acanthocephalan count and treatment group ($p = 0.391$). Removal of the acanthocephalan count by treatment group interaction term lowered the model AIC value by ∼12.1. No significant association was found between blood glucose and acanthocephalan count in this model (**Table 5** and **Figure 4**). Glucose levels in the 24SW and 72SW groups were significantly lower than in the 24FW group (**Tables 3**, **5**). This model also revealed that blood glucose was significantly negatively related to Fulton's K (**Table 5**).

DISCUSSION

We found little evidence that natural infection by acanthocephalan parasites affected the osmoregulatory performance of Atlantic salmon smolts immediately prior to, or within the first 72 h of, entry into salt water. Moreover, there was no evidence of physiological stress associated with acanthocephalan infection–at least as captured by blood glucose levels, which is one of several possible secondary physiological responses linked to general stress in fishes (Barton, 2002). Infection prevalence amongst experimental smolts was 66.2% and parasite abundance exhibited greater-than-Poisson variance (raw variance 10.3 times greater than raw mean), with few smolts containing many parasites (**Figure 2B**) and many individuals containing zero parasites: a common finding in parasitology in general (Poulin, 2007). The acanthocephalan

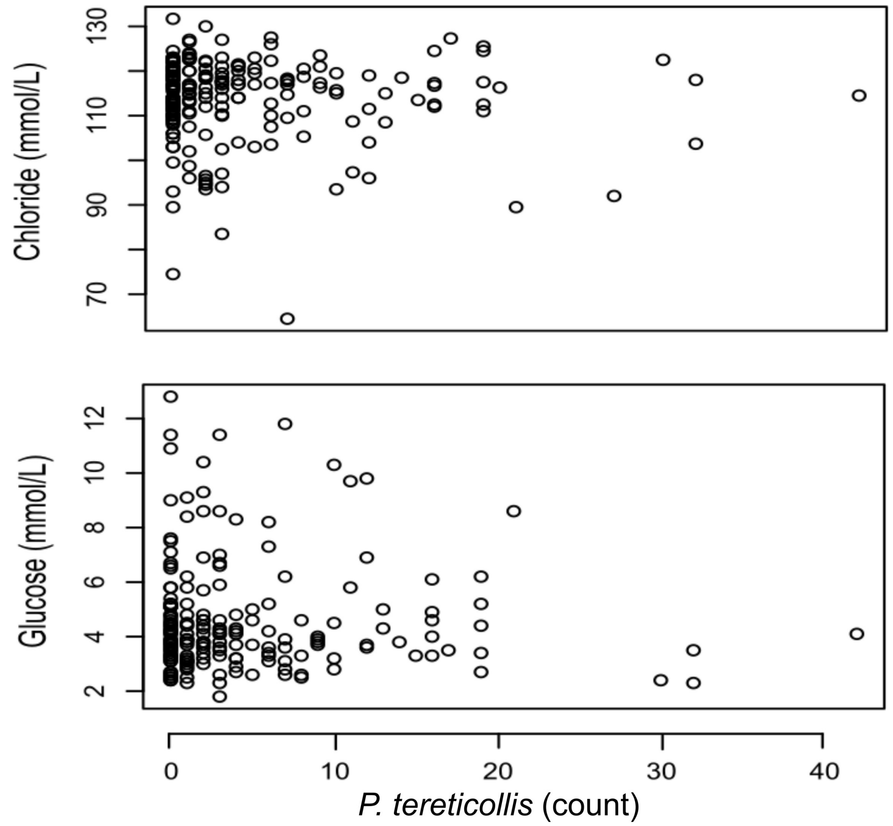

FIGURE 4 | Plasma chloride concentration (mmo/l) and blood glucose concentration (mmol/l) vs. *P. tereticollis* count for all smolts used in the experiment (n = 198).

TABLE 4 | Parameter estimates from the linear model where chloride was the response variable.

	Value	Std. Error	*t*-value	*p*-value
(Intercept)	116.899	6.076	19.241	<0.001
P. tereticollis	−0.060	0.053	−1.123	0.263
Treatment Group: 24SW	13.363	1.384	9.655	<0.001
Treatment Group: 72SW	14.998	1.389	10.796	<0.001
Fulton's K	−13.864	6.704	−2.068	0.040
Tank 2	−0.238	1.038	−0.229	0.819
Tank 3	−0.195	1.054	−0.185	0.854
Tank 4	−0.218	1.055	−0.207	0.836
Sex: Male	−0.198	0.773	−0.256	0.798
Operator: B	−0.354	0.730	−0.485	0.628

Intercept corresponds to females in treatment group 24FW in Tank 1 sampled by Operator A.

TABLE 5 | Parameter estimates from the linear model where glucose was the response variable.

	Value	Std. Error	*t*-value	*p*-value
(Intercept)	10.152	1.131	8.978	<0.001
P. tereticollis	0.013	0.009	1.422	0.157
Treatment Group: 24SW	−2.686	0.313	−8.575	<0.001
Treatment Group: 72SW	−3.481	0.316	−11.028	<0.001
Fulton's K	−3.743	1.224	−3.059	0.003
Tank 2	−0.193	0.183	−1.052	0.294
Tank 3	−0.134	0.186	−0.718	0.474
Tank 4	−0.167	0.193	−0.865	0.388
Sex: Male	−0.199	0.138	−1.439	0.152
Operator: B	−0.196	0.130	−1.509	0.133

Intercept corresponds to females in treatment group 24FW in Tank 1 sampled by Operator A.

parasites, which our DNA barcoding indicated to be *P. tereticollis*, consistently survived the first 72 h that smolts spent in salt water. However, although salinity in the saltwater tanks (∼26 PPT) was representative of local coastal waters, it was lower than in the open ocean habitat of the North Atlantic (∼35 PPT). This lower salinity may have made the environment in the saltwater tanks more tolerable for these acanthocephalan parasites than open ocean environments but was representative of local conditions experienced by these smolts in the wild in their first few days in coastal waters. However, given that the PTL1 strain of *P. tereticollis* (the only strain recorded in Ireland thus far) is believed to have co-evolved with salinity tolerant hosts (O'Mahony et al., 2004; Perrot-Minnot et al., 2018; Andreou et al., 2020), it is also possible that these acanthocephalans can survive for prolonged periods while their hosts are in open ocean environments.

The apparently widespread distribution of *P. tereticollis* (formerly called *P. laevis*) in Ireland (Hine and Kennedy, 1974; Munro et al., 1990; Molloy et al., 1993; Byrne et al., 2003), combined with the preferential use of salmonid definitive hosts by the PTL1 strain in Ireland (Pippy, 1969; Perrot-Minnot et al., 2018; Andreou et al., 2020; Tierney et al., 2020), implies that anadromous Irish salmonids often enter the marine environment while infected with these acanthocephalan parasites. Indeed, we found that infection prevalence amongst salmon smolts that were captured at the tidal limit of the Burrishoole catchment during the 2016, 2017, and 2018 smolt runs exceeded 65% in all years, with mean infection intensities of 9.2, 7.3, and 6.9, respectively. These infection rates are in line with those reported from other Irish catchments where *S. salar* and *P. laevis s.l.* coexist (Pippy, 1969), and previous studies have also found strong overdispersion in *P. laevis s.l.* abundance amongst host fish (Kennedy, 1974, 1996; Brown, 1989). Some authors have suggested that post-cyclic transmission (i.e., transmission occurring when a definitive host eats another definitive host) causes such acanthocephalan overdispersion patterns in definitive host species (Lassiere and Crompton, 1988; Valtonen and Crompton, 1990; Kennedy, 1999). However, the small body size and therefore presumably pre-piscivorous diet of the sampled smolts (no fish parts were observed in the stomach contents of the 175 smolts dissected at time of capture in 2016 and 2017) makes post-cyclic transmission from other host fish unlikely in this case. Given the extensive habitat heterogeneity within the Burrishoole catchment (ranging from fast-flowing streams to deep lakes) (Whelan et al., 1998), it is perhaps more likely that differing feeding behavior in areas with differing densities of the intermediate host *Gammarus duebeni* resulted in contrasting infection opportunities amongst these smolts.

Infection intensity amongst infected smolts was not associated with significant variation in any measure of smolt size (fork length, weight or Fulton's K). Infected males sampled in 2016 contained significantly more parasites per individual than infected females from the same year (12.8 vs. 4.9) although no sex bias in infection intensity was evident in 2017 or 2018. While no significant associations were found between infection prevalence and absolute measures of smolt size (i.e., fork length and weight), infected smolts were actually in better condition (at least as expressed by Fulton's K) than uninfected smolts. At first glance this is surprising, given that smolt condition might be expected to be negatively impacted by parasitic infection. However, salmon are known to undergo a rapid increase in length during smoltification that is not matched by an equivalent increase in weight, leading to a reduction in condition factor (Wedemeyer et al., 1980). Thus, if infection by *P. tereticollis* caused a reduction in growth (i.e., length gain) during the parr-smolt transformation period, it could account for the comparatively high condition factor observed amongst infected individuals relative to uninfected individuals. Alternatively, though, this finding could simply reflect the fact that pre-smolts that feed more actively in the weeks or months preceding their marine migration may attain increased condition relative to less active feeders but also have higher chances of consuming intermediate hosts (i.e., *G. duebeni*) infected with *P. tereticollis*, given that new infections of salmonids in Irish waters tend to peak in spring (Fitzgerald and Mulcahy, 1983; Molloy et al., 1995). Such seasonal patterns of parasite infections in salmonids are often associated with temporal changes in diet (Prati and Henriksen, 2020). Previous research has shown that many species of acanthocephalan parasites cause substantial damage to their hosts' intestines (Kim et al., 2011), reducing growth rates when infection intensities are high and leading to mortality in extreme cases (Latham and Poulin, 2002; Mayer et al., 2003). However, if many of the *P. tereticollis* found in the sampled smolts were relatively recent infections, there may have been insufficient time for their presence to cause a discernible effect on growth. As marine survival can be strongly associated with smolt size (Jonsson et al., 2017; Gregory et al., 2018), any parasite-induced impact on growth or condition factor is likely to have fitness consequences.

We found that *P. tereticollis* deeply penetrated all layers of their host's intestinal wall with their praesoma (hooked proboscis), and in some cases even penetrated the peritoneum and adjacent muscle wall, passing completely out of the intestines in the process (**Figure 2A**), similar to previous reports (Dezfuli et al., 2002a). The anchoring method used by *P. laevis s.l.* has been shown to destroy intestinal mucosa (Wanstall et al., 1988), eliciting a localized inflammatory response (Wanstall et al., 1986; Dezfuli et al., 2008, 2011) and copious mucus secretion (Harris, 1972; Dezfuli et al., 2016). Although there is only limited evidence indicating that salmonids infected with *P. laevis s.l.* suffer reduced growth (Wanstall, 1984), it appears that these infections may cause modifications to the physiological functioning of their host's alimentary tract, potentially reducing control of transepithelial ion transport (Dezfuli et al., 2002a). Thus, we expected that negative impacts from *P. tereticollis* infection might only manifest when the host entered salt water, where effective osmoregulation requires efficient control of transepithelial ion transport by the intestine (Whittamore, 2012).

As anticipated (i.e., Bowers et al., 2000; Urke et al., 2014; Stewart et al., 2016), plasma chloride concentrations were significantly higher in smolts sampled after 24 and 72 h in salt water than in smolts sampled after 24 h in fresh water. However, *P. tereticollis* abundance was not associated with variation in chloride levels in any treatment group, indicating that any intestinal damage caused by *P. tereticollis* was insufficient to cause hyper or hypo-osmoregulatory failure. The range of plasma chloride concentrations in the freshwater and saltwater treatment groups were similar to those reported from other studies of Atlantic salmon in freshwater and saltwater environments (Oppedal et al., 1999; Wells et al., 2006; Kolarevic et al., 2014). However, no sampled smolts displayed highly elevated chloride levels in line with levels that have been recorded in salmon smolts infected with high numbers of salmon lice (*L. salmonis*), which would indicate compromised hypo-osmoregulatory function (Grimnes and Jakobsen, 1996; Wagner et al., 2003).

Hyperglycemia (elevated blood glucose) is a secondary stress response in fish that has been widely used as an indicator of parasite-induced stress in smolts (Wagner et al., 2003; Finstad et al., 2007; Long et al., 2019). No association between glucose and *P. tereticollis* count was detected in any of the treatment groups, which would suggest that *P. tereticollis* infection intensities observed in this study were insufficient to cause a discernible stress response in smolts in hypo-osmotic environments or within 24- to 72-h of entry into hyper-osmotic environments. However, blood glucose is just one of many possible indicators of a complex, multi-dimensional stress response and thus it remains possible that other measures such as plasma cortisol could reveal parasite-induced effects on host stress levels that we were unable to detect.

Glucose levels were lowest in the group sampled after 72-h in salt water, which might relate to the longer period of fasting that this group experienced prior to sampling (72 h vs. 24 h in the other treatment groups). Plasma glucose is known to be affected by the feeding history and metabolic status of fish (Wells et al., 2006) and so it is possible that the comparatively low glucose levels found in the 72SW treatment group resulted from increased caloric deficit.

The results of this study are based on the use of four replicate tanks for each treatment group. However, despite having 66 smolts in each treatment group (i.e., moderate to large sample sizes), only a small number of smolts had high infection intensities (**Figure 1**), and this may have reduced our ability to detect subtle parasite-induced changes in the blood parameters that we investigated. Also, all *P. tereticollis* found in each treatment group were alive and the vast majority were securely anchored to the intestinal wall by their praesomae, with no sign that others had recently detached (i.e., no vacant perforations in the intestinal wall). When *P. tereticollis* are anchored to the intestinal wall, their praesoma and inflated proboscis bulb appear to form an effective plug, preventing movement of liquid through the surrounding intestinal perforation. The *P. tereticollis* that parasitize juvenile salmon in the Burrishoole catchment are generally absent from adult salmon when they return to the river system for spawning (Deirdre Cotter, pers. obs.). Similarly, Pippy (1980) found no *P. laevis s.l.* in adult salmon caught off Greenland, concluding that the parasites could not endure the prolonged migration period of their host. Furthermore, Molloy et al. (1993) found that sea trout returning to the Burrishoole catchment had lower infection prevalence and intensity than were found in emigrating trout smolts, indicating that *P. tereticollis* are lost during the marine migration. Given that the lifespan of *P. laevis s.l.* in freshwater barbel (*Barbus barbus*) hosts has been estimated at between 6 and 8 months (Nachev and Sures, 2016), it is likely that *P. tereticollis* generally die before migrating salmon hosts return to fresh water. Presumably, after some period in the marine environment these acanthocephalans detach from their hosts' intestines, leaving intestinal perforations unplugged and thereby potentially facilitating ingress of salt water into the peritoneal cavity. However, as *P. tereticollis* in the sampled smolts remained alive and attached after 72 h in salt water we were unable to investigate this possible delayed pathology. As suggested by Pippy (1969), it would be useful for future studies to accurately establish the lifespan of *P. laevis s.l.* in Atlantic salmon at sea.

The shifts between freshwater and marine environments that define diadromous fishes are inherently stressful, demanding complex physiological responses from migrants that are simultaneously exposed to unfamiliar predators, parasites and pathogens. Mortality rates during this transitional period can be particularly high, and any pre-existing factor that increases stress or interferes with physiological processes such as osmoregulation may compromise long-term survival at sea (Finstad et al., 2012, 2007; Hostetter et al., 2012, 2011). While the contribution of non-lethal stressors to delayed marine mortality is often difficult to detect, particularly in cases where multiple stressors have a cumulative effect, their impacts are likely to play an important role in determining the performance of anadromous populations. We have shown that a high proportion of wild Atlantic salmon smolts entering the marine environment from the Burrishoole catchment in recent years are infected with *P. tereticollis* and, based on the literature, it appears likely that similar infection rates of smolts are common in Ireland but not elsewhere. However, we found no evidence to indicate that the infection intensities observed amongst the sampled smolts were associated with altered osmoregulatory performance or blood glucose levels in freshwater or saltwater environments. Despite this, it is possible that infection by *P. tereticollis* causes pathologies that we did not test for or that occur later in the marine environment as salinity increases or as the parasites detach from the intestine. Given the high prevalence of *P. tereticollis* infection amongst anadromous salmonid populations in Ireland, it would be valuable to investigate whether such delayed parasite-induced pathologies occur at sea. Such an investigation could be undertaken in a similar manner to the present study, but conducted over a longer time period at a higher salinity, potentially providing results with applied relevance in the context of fishery management and aquaculture (e.g., marine rearing of farmed Atlantic salmon).

In conclusion, our study adds to a growing body of work examining ecophysiological processes underpinning the performance of migratory fishes (McCormick et al., 1998, 2009; Hinch et al., 2005; Cooke et al., 2008, 2012; Groot, 2010; Björnsson et al., 2011; Eliason and Farrell, 2016) and draws particular attention to the potential role of parasites in determining osmoregulatory abilities. The physiological impacts of parasitism often depend on the extent and patterns of historical coevolution between hosts and their parasites (Prenter et al., 2004; Britton et al., 2011; but see Lymbery et al., 2014). Migratory species are particularly interesting in this context as they are exposed to native and non-native parasites in multiple geographic locations and habitat types. Consequently, infection or infestation may have delayed fitness consequences that occur in a different habitat to where the parasites are initially encountered. We therefore encourage further ecophysiological work on the impacts of parasites on the performance of migratory animals across variable and rapidly changing environments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Marine Institute Animal Welfare Committee (MI Establishment Authorisation No: AE19121) and the Health Professionals Regulatory Authority (HPRA Classification Request Number: 066).

AUTHOR CONTRIBUTIONS

RF, TR, and RP conceived and designed the experiment. RF, RP, and GR collected the data. ED conducted the genetic barcoding. RF and TR analyzed the data. RF wrote the first draft of the manuscript. All authors contributed to drafts of the manuscript and gave final approval for publication.

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Unraveling the World's Longest Non-stop Migration: The Indian Ocean Crossing of the Globe Skimmer Dragonfly

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Insect migration redistributes enormous quantities of biomass, nutrients and species globally. A subset of insect migrants perform extreme long-distance journeys, requiring specialized morphological, physiological and behavioral adaptations. The migratory globe skimmer dragonfly (*Pantala flavescens*) is hypothesized to migrate from India across the Indian Ocean to East Africa in the autumn, with a subsequent generation thought to return to India from East Africa the following spring. Using an energetic flight model and wind trajectory analysis, we evaluate the dynamics of this proposed transoceanic migration, which is considered to be the longest regular non-stop migratory flight when accounting for body size. The energetic flight model suggests that a mixed strategy of gliding and active flapping would allow a globe skimmer to stay airborne for up to 230–286 h, assuming that the metabolic rate of gliding flight is close to that of resting. If engaged in continuous active flapping flight only, the flight time is severely reduced to ∼4 h. Relying only on self-powered flight (combining active flapping and gliding), a globe skimmer could cross the Indian Ocean, but the migration would have to occur where the ocean crossing is shortest, at an exceptionally fast gliding speed and with little headwind. Consequently, we deem this scenario unlikely and suggest that wind assistance is essential for the crossing. The wind trajectory analysis reveals intra- and inter-seasonal differences in availability of favorable tailwinds, with only 15.2% of simulated migration trajectories successfully reaching land in autumn but 40.9% in spring, taking on average 127 and 55 h respectively. Thus, there is a pronounced requirement on dragonflies to be able to select favorable winds, especially in autumn. In conclusion, a multi-generational, migratory circuit of the Indian Ocean by the globe skimmer is shown to be achievable, provided that advanced adaptations in physiological endurance, behavior and wind selection ability are present. Given that migration over the Indian Ocean would be heavily dependent on the assistance of favorable winds, occurring during a relatively narrow time window, the proposed flyway is potentially susceptible to disruption, if wind system patterns were to be affected by climatic change.

Keywords: insect migration, migratory physiology, wind trajectory analysis, dragonfly migration, *Pantala flavescens*

INTRODUCTION

Insect migration is a widespread natural phenomenon responsible for the distribution of thousands of tons of biomass and trillions of individuals each year (Hu et al., 2016; Florio et al., 2020). The annual and seasonal migration of insects translocates nutrients and ecological interactions, and has enormous influence on essential ecosystem functions such as pollination, herbivory and predation (Chapman et al., 2004; Krauel et al., 2015; Semmens et al., 2018; Wotton et al., 2019; Satterfield et al., 2020). These influences can occur over considerable ecological scales, as many insects are capable of long-distance migration covering thousands of kilometers and involving several generations (Zhan et al., 2011; Hallworth et al., 2018; Talavera et al., 2018; Gao et al., 2020a).

Migration in the insect order Odonata (dragonflies and damselflies) is relatively poorly studied (May, 2013), in spite of these insects' conspicuousness and popularity (May, 2019). However, technological developments and an increasing interest in the use of stable isotope analysis has recently resulted in several noteworthy studies into the migratory dynamics of dragonflies (Hallworth et al., 2018; Knight et al., 2019; Borisov et al., 2020a; Hobson et al., 2021). Interestingly, potentially the longest non-stop migration of any dragonfly, indeed of any terrestrial animal when controlling for body length or mass (see **Supplementary Table 1**), is the proposed multi-generational, transoceanic migration of the globe skimmer dragonfly (*Pantala flavescens*), which has been postulated to migrate from India to Africa and back again (Anderson, 2009; Hobson et al., 2012).

At first consideration, a flight of > 2,000 km across the open ocean by an insect just 5 cm long and weighing just 300 mg may appear unlikely. Yet, there are numerous reports of the globally distributed globe skimmer being encountered at sea, and it has reached many isolated islands (see below), indicating that this species is not deterred from crossing large bodies of water, unlike other dragonflies (Wikelski et al., 2006). Monitoring of nocturnal insect migration over the Bohai Sea in China has recorded the species extensively (Feng et al., 2006), demonstrating its capacity for flying at least 150–400 km over the sea during a single night. Sightings hundreds of kilometers offshore were also noted by seafarers during the nineteenth and early twentieth centuries (Asahina and Turuoka, 1968; McLachlan, 1896; Davidson, 2012), and more recently, entered into online observation platforms by sailors (iNaturalist, 2018). The circum-tropical distribution of the globe skimmer, with individuals found on remote islands in the Pacific and Indian Oceans (Gibson-Hill, 1950; Samways, 1998; Samways and Osborn, 1998; Buden, 2010, 2018; Carr, 2020), provides further support to the idea that this species is capable of regularly carrying out long, cross-ocean flights. However, the most rigorous evidence of a re-occurring, seasonal transoceanic migration comes from regular monitoring of the globe skimmer's annual mass appearance and departure from the Maldives, 430 km from the Indian sub-continent (Anderson, 2009). This hypothesized annual return migration between India and Africa has been frequently cited by researchers (Web of Science 80 peer-reviewed citations, as of 10th July 2021), however, the arrival of Indian globe skimmers in Africa in the autumn, as well as of African globe skimmers in India in the spring, has yet to be confirmed. Analyses investigating the eco-physiological dynamics and actual feasibility of the migration are also lacking.

Extreme endurance and long-distance migration in animals are subjects of considerable interest. Alongside the awe-inspiring journeys of long-distance migrants, such as the 8 days non-stop flight of the bar-tailed godwit *Limosa lapponica* (Gill et al., 2009), unraveling the complex adaptations necessary to accomplish such extreme feats of endurance is also central for our basic understanding of animal physiology, conservation and evolution, with further relevance for applied fields such as engineering and health (Hedenström, 2010; Jenni-Eiermann et al., 2014; Cooper-Mullin and McWilliams, 2016; Cheeseman et al., 2018). Bird migrants have been studied extensively, and individual-level studies in particular have revealed migratory adaptations, but until certain types of trackers are small and light enough to be carried by animals of < 1 g, migration in insects generally must be inferred remotely, via tools such as radar, modeling and stable isotopes.

One remote sensing tool that has been instrumental in the study of insect migration, is wind trajectory analysis. Wind patterns have pronounced effects on the movement of all aerial migrants (Liechti and Bruderer, 1998; Åkesson and Hedenström, 2000; Chapman et al., 2015), and similarly to birds, insects can utilize seasonally favorable winds for migration (Chapman et al., 2008, 2010). Indeed, depending on their size and migratory distance, they may rely solely on winds for their migration (Reynolds et al., 2016). Wind trajectory analysis can simulate the potential origins and destinations of insect migrations by input of regional and time specific wind data, while also allowing for species-specific parameters of migratory behavior. For example, it can be used to assess invasion risk of new pest species (Li et al., 2019; Wang et al., 2020), trace the likely departure point for specific migration events (Kim et al., 2010) and reconstruct dispersal pathways (Lander et al., 2014).

In the Indian Ocean region, the trade winds converge at an atmospheric "front" known as the Inter-Tropical Convergence Zone (ITCZ), which shifts seasonally, and influences rainfall and climate in association with monsoonal circulation. In autumn, the ITCZ moves south with winds blowing from the NNE from India, and in spring the ITCZ migrates northwards, shifting wind direction to India from the SSW (Barry and Chorley, 1992). Globe skimmers, as well as avian migrants, are believed to utilize these seasonal shifts in wind direction for their migration over the Indian Ocean (Moreau, 1938; Skerrett, 2008; Anderson, 2009).

Alongside adaptations pertaining to wind usage, insects performing long-distance migration may require specific adaptations in physiology and behavior, such as the ability to accumulate fat for energy storage (Blem, 1980; Hedenström, 2010), performing energy efficient flight (Gibo, 1981) and accurate navigational skills (Mouritsen and Frost, 2002; Dreyer et al., 2018). Dragonfly physiology has been studied extensively in the past (Kallapur and George, 1973; May, 1979, 1984, 1995), and considering the extreme energetic challenges facing a < 1 g migrant aiming to cross an ocean, we may use this knowledge to test how known physiological parameters can assist in the understanding of the feats and limitations of globe skimmer migration.

Here, we investigate the proposed multi-generational, long-distance transoceanic migration of the globe skimmer using energetic flight modeling and wind trajectory analysis. Energetic flight models apply estimates of metabolic rate, physiology and behavioral ecology to calculate theoretical flight durations and distances, while wind trajectory analyses simulate the possibility of wind-assisted migratory passage at different heights, departure sites and seasons. Specifically, we will address the following questions: (i) How long and how far can a globe skimmer stay airborne if just relying on accumulated body fat? (ii) Do favorable winds occur over the Indian Ocean that may be utilized for transoceanic migration by the globe skimmer? (iii) Where would a globe skimmer relying on wind assistance arrive? (iv) How long would a transoceanic migration relying on wind assistance take?

MATERIALS AND METHODS

Study Species

The globe skimmer or wandering glider (*Pantala flavescens*, Libellulidae, **Figure 1**) is an obligate migrant dragonfly, believed to be specialized in utilizing seasonal wind systems to move between habitats where temporary rain pools are available (Corbet, 2004). It is widely distributed across all continents except Europe and the polar regions, and is usually common where it occurs (Corbet, 2004). The globe skimmer has a comparatively fast nymphal development time for a dragonfly, of approximately 40–60 days depending on water temperature (Van Damme and Dumont, 1999; Suhling et al., 2004; Ichikawa and Watanabe, 2016).

FIGURE 1 | Globe skimmer (*Pantala flavescens*). Photo by: Johanna Hedlund 2019.

The globe skimmer is common in India, and occurs and breeds year-around in the very south (Corbet, 1988; Muthukumaravel et al., 2015). In the central part of India it peaks in abundance during the post-monsoon period (September–December), disappearing until the next heavy rains in May–July (Corbet, 1988; Sharma and Joshi, 2007; Kulkarni and Subramanian, 2013). The species is not resident in the Maldives, but starts to occur during migratory passage in October, peaking in November–December, with numbers trailing off in January–February, and with a small re-occurrence around May (Anderson, 2009; Hobson et al., 2012).

Globe skimmers have a mean body length of 50 cm and an approximate wingspan of 80 mm (Su et al., 2020). Individuals of the species typically weigh ∼300 mg (May, 1979, 1981; Ishizawa, 2007; Su et al., 2020), but mass ranges between 228 mg in small males and 553 mg in large females (Ishizawa, 2007). The anatomy of the globe skimmer indicates its adaptation for long, migratory flights. The surface of dragonfly wings is corrugated, an adaptation shown to counteract turbulence and facilitate lift (Obata and Shinihara, 2009) and the wings of globe skimmers in particular, present specialized anti-fatigue properties that are beneficial when alternating load during flight (Li et al., 2014). The globe skimmer has an expanded anal margin and broad hindwings with lobes, both features characteristic of species adapted to migration and gliding flight (Hankin, 1921; Corbet, 1962; Miller, 1962; Grabow and Rüppell, 1995; Johansson et al., 2009). The globe skimmer also has specialized thoracic musculature, which is assumed to improve long-distance flight (Bäumler et al., 2018).

Physiology of Long-Distance Migration

To assess the physiological potential and constraints of globe skimmer migration, parameters of the species ecology, behavior and physiology were obtained from the literature. Specifically, empirical data was sought on (i) species-specific flight speeds at different flight modes, in order to estimate distance that could be traveled; (ii) metabolism and energy storage capacity, in order to estimate energy expenditure at different flight modes and the distance that could be traveled on certain amounts of accumulated fat; and (iii) environmental and behavioral factors affecting these features, i.e., to identify species tendency to use or alternate between different flight modes and whether certain conditions, e.g., temperature, would influence flying capacity or flight mode. The information gathered was then entered into an energetic flight model (see below).

Flight Speed

Dragonfly flight is aerodynamically advanced, and great maneuverability and different flight modes can be achieved by the precise angling and independent motion of the fore- and hindwings (Salami et al., 2019; Liu et al., 2021). When migrating, globe skimmers have been observed to travel at velocities of 3.1–7 m/s depending on tailwind (Srygley, 2003; Feng et al., 2006). Also known as the wandering glider, the globe skimmer is often observed engaging in gliding flight for long periods of time (Hankin, 1921; Scorer, 1954; Corbet, 2004), and using thermal lift for soaring (Gibo, 1981). During gliding and soaring, the globe skimmer's wings are outstretched but immobile, suggesting that energy expenditure is very low, potentially nearing that of resting metabolism (Gibo, 1981). However, to counteract instability and rolling movements when gliding, dragonflies hold their wings in a shallow dihedral (Wakeling and Ellington, 1997), which suggests that some energy is utilized to keep the wings in position. Studying gliding speeds in Odonates, Wakeling and Ellington (1997) found that prolonged gliding speeds outside the range of 0.6–0.9 m/s should not be aerodynamically efficient, as energy expenditure would increase because of drag. However, the authors did record speeds ranging from 0.5 to 2.6 m/s, and it is known that gliding dragonflies tend to combine gliding with shorter bouts of flapping (pers. obs.; Wakeling and Ellington, 1997; Gibo, 1981; Obata et al., 2014), so as to maintain altitude and speed when little wind is available to uphold gliding.

Metabolism

When estimating energy expenditure during rest in a globe skimmer weighing 330 mg, May (1979) recorded a metabolic rate of 0.0024 W (W = joules/s). For other behaviors, such as active flying, metabolic rate is only available for other species. In the green darner (*Anax junius*), which weighs ∼1,000 mg, active feeding flight is achieved at 0.26–0.43 W (May, 1995). Long-range migratory flights in dragonflies should predominantly be achieved through gliding, as this flight mode uses less energy (Gibo and Pallett, 1979; Gibo, 1981; Corbet, 2004; Vargas et al., 2008). When flight mode type was scored in migrating monarch butterflies, they were observed to soar 83.5% of the time, and only to engage in powered active flight 15.3% of the time (Gibo and Pallett, 1979). A high proportion of time spent gliding was also observed in migrating dragonflies, in relation to other flight modes (May, 1995). Unfortunately, the metabolic rate of gliding flight in dragonflies is not known empirically, but when measured in birds, has been shown to be similar to baseline energy consumption (Duriez et al., 2014), or twice as high (Sapir et al., 2010).

Fueling Flight

Similar to birds, insects are known to gain body mass by fat accumulation in preparation for migration (Blem, 1980; Corbet, 1984; Weber, 2009) and experiments on globe skimmers have shown that prolonged flight decreases lipid reserves (Kallapur and George, 1973). However, actual estimation of fat content in dragonflies, measured in species actively migrating, is absent from the literature, with one exception. During autumn migration in North America, it was estimated that up to 27% of the body mass in the green darner dragonfly can consist of fat reserves (May and Matthews, 2008). This fat percentage places dragonflies in the middle of the range measured in other migrant insects (Blem, 1980; Brower et al., 2006). Importantly, all fat stored in an insect's body will not be available as fuel during migration, as lipids are essential for other physiological functions, for example cell membrane structure and reproduction (Arrese and Soulages, 2010).

Energetic Flight Model

An energetic flight model was constructed based on the available information presented above. All simulations were run considering a standard individual of 330 mg containing 27% fat, of which 90% was available for use to power flight (with the remaining 10% reserved for other physiological functions). All energy consumption for flight was assumed to be performed through the burning of fat, at the rate of 38 kJ/g (Berg et al., 2002). Two flight modes requiring different rates of energy consumption were considered. Active flapping flight was set at a rate of 0.19 W, inferred from the regression in Figure 7 of May (1995) for similarly sized dragonflies. Gliding flight was set at a rate of 0.002904 W, which is the resting metabolic rate in a 330 mg globe skimmer (0.0024 W, May, 1979) increased by 20%. There is no reference for the metabolic rate for gliding in the literature, but as it is suggested to be near to that of resting (Gibo, 1981), but still has to account for energy required to angle the wings optimally (Wakeling and Ellington, 1997; Obata et al., 2014), we chose an increase of 20%.

Three metrics were calculated: (1) total flight duration assuming active flight, gliding flight and a combination of active and gliding flight modes (80% gliding, 20% active flight); (2) total flight distance assuming no wind assistance; and (3) total flight distance with wind assisted flight. Flight duration analysis and distance modeling were performed using MATLAB R2019a. A flowchart visualizing the input data and resulting estimations can be viewed in **Supplementary Figure 1**.

Wind Trajectory Analysis

To simulate possible migratory routes of globe skimmers departing on autumn and spring migration across the Indian Ocean, we developed a numerical trajectory model that considered flight behavior and self-powered flight vectors (see for example Wu et al., 2018). The trajectory calculations were driven by high spatio-temporal resolution weather conditions, simulated by the Weather Research and Forecasting (WRF) model (version 3.8)[1]. The WRF is developed for research on actual or idealized atmospheric conditions, and is well adapted for meso-scale numerical forecasting applications. In this study, the dimensions of our trajectory model domain were 160 × 200

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[1] www.wrf-model.org

grid points at a resolution of 60 km. Thirty-two vertical layers were available and the model ceiling was 100 hPa. The model was set to adhere to the following rules: (i) trajectories were enforced to follow the wind direction downwind at all times; (ii) if the trajectories experienced temperatures under 5°C they were suspended; (iii) if windspeeds < 5 m/s were experienced, the trajectories compensated with simulated self-powered flight to uphold a minimum speed of 5 m/s.

Three departure sites for the model were set for autumn, spanning the western seaboard of the Indian subcontinent and the Maldives: (1a) Gwarka lighthouse, Gujarat, northern India (22° 14′ 18.5922″, 68° 57′ 34.3902″); (1b) Vasco da Gama, Goa, western India (15° 23′ 9.7182″, 73° 50′ 38.544″); and (1c) Malé, the Maldives (4° 6′ 10.5408″, 73° 18′ 11.5086″). The Maldives was chosen as it is the only location in the region where regular migratory movements of globe skimmers have been documented and where monitoring has been performed long-term (Anderson, 2009). The remaining two sites were chosen to acquire evenly spaced departure sites at additional locations along the Indian western coastline. As wind speeds typically increase with height above ground, insect migration that does not rely solely on self-powered flight often takes place above the flight boundary layer at high altitude (Wilson, 1995; Reynolds et al., 2016), and thus the trajectories were initiated at three different heights: 500, 1,000, and 1,500 m. Trajectories were run for a simulation period of 10 days until suspended, starting once every day at 07.00 local time for all days during the period September–December 2019, generating in total 1,098 migration events.

Two departure sites were set for the model for spring: (2a) Guardafui lighthouse, Somalia (11° 45′ 3.2394″, 51° 15′ 21.2394″); and (2b) Shakani, Kenya (−1° 39′ 39.6″, 41° 33′ 29.5194″). Trajectories were again initiated at three heights (500, 1,000, and 1,500 m) and were run for 6 days until they were suspended (they were run for less time than the autumn simulations, as the eastward migration was found to take considerably less time than the westwards migration), starting once every day at 07.00 local time for all days during the period May–July in 2019, generating in total 366 migration events.

RESULTS

Energetic Flight Model

When engaged in 100% active, flapping flight (metabolic rate of 0.19 W), the energy reserves of a standard individual globe skimmer last just 4.39 h, in stark contrast to 286.96 h if engaged only in gliding flight (metabolic rate of 0.002904 W). Considering a combination of flight modes (Gibo and Pallett, 1979), consisting of 20% active flight and 80% gliding flight, the corresponding flight duration is 230.45 h, which is equivalent to 9.6 days (**Supplementary Figure 1**). Importantly, these are the absolute maximum flight durations, estimated at the limit of energetic capacity, whereby a standard individual transforms 90% of its stored lipids into movement energy. This caveat must also be kept in mind for estimates of flight distance.

Flight distance was estimated for a standard individual employing self-powered flight consisting of a combination of flight modes (20% active flight and 80% gliding flight) under self-powered speeds of 1.4, 1.8, and 2.6 m/s (**Figure 2** and **Supplementary Figure 1**). These three speeds are based on the assumption of a speed of 5 m/s for active flight (20% of the time) and three differing gliding speeds (80% of the time): 0.5, 1, or 2 m/s. Gliding speeds without wind assistance have been suggested to be aerodynamically effective only in the range of 0.6–0.9 m/s (Wakeling and Ellington, 1997), thus our highest modeled gliding speed (2 m/s) may be in excess of gliding speeds obtainable without wind assistance. Flight distance was also estimated for a standard individual assisted by differing wind speeds ranging from 1 to 15 m/s (**Figure 3**).

The minimum distances a globe skimmer would have to cover to cross the Indian Ocean, from any of the departure sites used in the wind trajectory analyses (**Table 1**), are given the IDs Autumn 1–3 (938, 1,744, and 2,536 km) and Spring 1 (2,430 km), and used to illustrate globe skimmer migratory capacity in **Figure 2** and **Supplementary Figure 2**. (Note here that only one departure site is considered in spring, the reason for this is further explained in the section on the results of the wind trajectory analysis). The shortest distance, Autumn 1 (938 km), is within the 230.45 h flight duration threshold for all three assumed self-powered flight speeds of a standard individual employing a combined active-gliding flight (**Figure 2**). However, only a speed of at least 2.6 m/s would ensure that a standard individual can cover the distance of "Autumn 2" (1,744 km) within the maximum flight duration of 230.45 h, with the two remaining distances (2,536 and 2,430 km) taking longer than this upper threshold when relying on self-powered flight alone.

We find that a windspeed of as low as 1.5 m/s is sufficient to enable a standard individual to cross the shortest proposed distance over the Indian Ocean, Autumn 1 (**Figure 3**), while tailwinds of at least 2.5–4 m/s are required to cross the longest distance, Autumn 3 (**Figure 3**). Natural conditions may not provide consistent windspeeds for many hours, or favorable wind assistance in the optimum direction. These nuances are clear from the results of our wind trajectory analyses (see below, **Figures 4–9**). Thus, the assistance of greater wind speeds may be necessary. In the section below on the results of the wind trajectory analysis, we report that simulated migration events in fact are assisted by winds > 4 m/s (e.g., mean wind speed across all sites and heights = 6.0 m/s in autumn and 9.7 m/s in spring).

Wind Trajectory Analysis

A total of 1,098 trajectories were run to simulate autumn migratory flights across the Indian Ocean (**Figures 3–5**), equating to one trajectory for each starting height (500, 1,000, and 1,500 m), for each of the three departure sites, every day during the specified time window. An autumn trajectory was deemed successful if it reached any country in Africa, the Arabian Peninsula or Iran. A total of 180 trajectories fulfilled these requirements, but 13 were removed as they experienced temperatures under 10°C (due to high altitude), which is presumed to be too close to a temperature threshold where flight can be adequately sustained (Corbet, 1957; Rowe and Winterbourn, 1981; Dumont, 1988; Sformo and Doak, 2006). The remaining successful trajectories ($N = 167$, 15.2%) took on

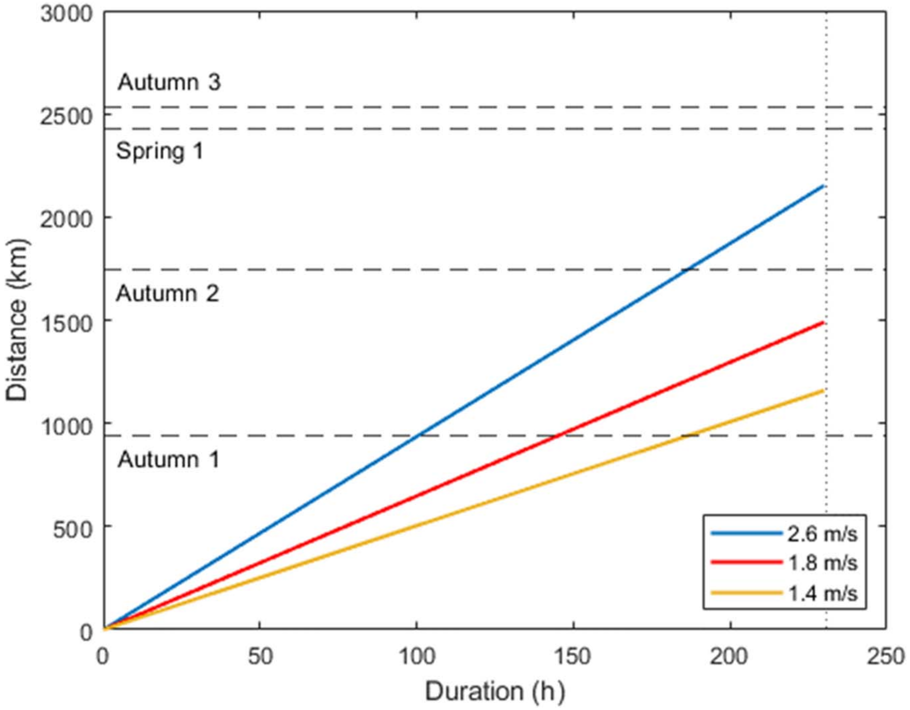

FIGURE 2 | Estimated flight distances assuming a combination of 20% active flight and 80% gliding flight. Colored lines reflect different self-powered speeds, and the figure assumes no contribution of wind. Self-powered speeds are based on the assumption of 5 m/s for active flight (20% of the time) and three differing gliding speeds (80% of the time): 0.5, 1, and 2 m/s. The dotted vertical line denotes the estimated maximum flight duration (230.45 h). The dashed horizontal lines reflect hypothetical flight distances during autumn and spring migration, based on the shortest distance from the wind trajectory departure sites to land across the Indian Ocean (Autumn 1: 938 km; Autumn 2: 1,744 km; Autumn 3: 2,536 km; and Spring 1: 2,430 km, see **Table 2**).

average 127.2 h ± 3.8 to cross the Indian Ocean (**Figures 7A–C**). Separated by departure sites, a transoceanic crossing took on average 114.2 h ± 4.5 from Gwarka lighthouse (Gujarat, northern India), 146.82 h ± 6.6 from Vasco da Gama (Goa, western India), and 164.22 h ± 10.23 from Malé, the Maldives (**Table 2**). Thus, flight time was in general shorter for trajectories setting out from Gwarka lighthouse, and the longest for trajectories setting out from the Maldives, which correlates with the distance required to cross the ocean to land in each case. The departure site at Gwarka lighthouse also had the greatest number of successful trajectories (*N* = 105, 62% of all successful), followed by the departure site in Vasco da Gama (*N* = 53, 31%) and lastly The Maldives (*N* = 9, 5%). The arrival sites of all successful trajectories in autumn are illustrated in **Figures 7A–C**. Most autumn wind trajectories reached land during December (*N* = 67), followed by November (*N* = 49), October (*N* = 34), January (*N* = 15), and September (*N* = 2). Note that arrival in January 2020 is due to the fact that trajectories starting late dates in December, running for 10 days, would finish their routes the following month. The mean wind speed across all autumn trajectories was 6.0 m/s, with a slightly higher speed measured for the departure sites of Gwarka lighthouse and Vasco da Gama (6.1 m/s), followed by The Maldives (5.9 m/s).

During spring migration, a total of 366 trajectories were run for each departure site, equating to one trajectory for each starting height (500, 1,000, and 1,500 m), every day during the specified time window (**Figure 8**). However, as the trajectories departing from Kenya stayed over land and did not cross the ocean until they reached Somalia, hence overlapping with the Somalian trajectories (**Supplementary Figure 2**), the Kenyan trajectories are not discussed further. A spring trajectory was deemed successful if it reached Pakistan, India, Sri Lanka or the Maldives. A total of 162 of 366 spring migration trajectories departing from Somalia were successful in crossing the Indian Ocean and reaching land, but of these 12 were removed as they experienced temperatures too low for flight (<10°C), leaving 150 successful trajectories (40.9%) (**Figure 9**). Successful spring trajectories took on average 55.24 h ± 1.6 to reach land (**Table 2**), a considerably shorter amount of time than required during the simulated autumn migration. Notably, spring trajectories that reached land displayed geographical segregation based on starting altitude (**Figure 9**), with those initiated at 500 m predominantly arriving to Pakistan and northern India, and the higher starting altitudes progressively being distributed southwards along the western Indian coastline. Most spring wind trajectories reached land during July (*N* = 80), followed by June (*N* = 56), May (*N* = 11), and August (*N* = 3). Note that arrival in August is due to the fact that trajectories starting late dates in July, running for 6 days, would finish their routes the following month. The mean wind speed across all spring trajectories departing from Somalia was 9.7 m/s, considerably faster than the mean estimated for the autumn crossing.

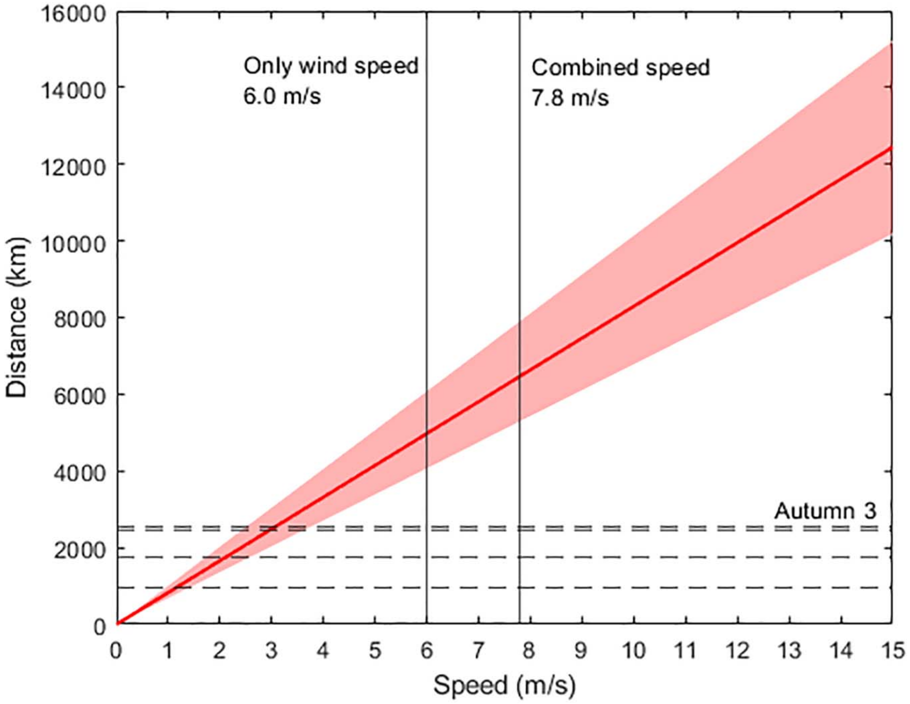

FIGURE 3 | Estimated flight distance assuming a combination of unaided flight and wind. The red line reflects a standard individual, and the shaded area reflects simulated variation of ± 10% in input variables (energy available and metabolic rate). The horizontal dashed lines denote various flight distances during the autumn and spring migration (Autumn 1: 938 km; Autumn 2: 1,744 km; Autumn 3: 2,536 km; and Spring 1: 2,430 km, see **Table 2**), with the longest migration labeled (Autumn 3: 2,536 km). The vertical, solid lines denote mean wind speed during the autumn migration based on wind trajectory analyses (6 m/s), and mean wind speed during the autumn migration with the addition of the mean unaided flight speed (1.8 + 6 = 7.8 m/s).

DISCUSSION

Globe skimmer departure from the Asian continent toward The Maldives, and the subsequent passage across Maldives, are well-documented (Anderson, 2009; Hobson et al., 2012). However, whether this represents a regular, deliberate, transoceanic migration from India to Africa in the autumn, followed by a return migration in spring, is yet to be fully confirmed. Here, we have investigated the physiological, environmental and behavioral factors that govern the feasibility of this proposed migration. We posed four questions to frame our investigation. We applied an energetic flight model to answer the first question, which queried how long a globe skimmer could stay airborne and how far it could travel if relying solely on accumulated fat and self-powered flight (i.e., without wind assistance). The results suggest a wide range of ∼4–287 h for globe skimmer flight duration, depending on flight mode metabolics, and that a maximum distance of ∼2,250 km could be flown, given exceptionally favorable conditions, and a high self-powered gliding speed of 2.6 m/s. In summary, these measurements confirm that a self-powered migration over the Indian Ocean is possible, but only if it would take place at crossings less than 2,250 km and with exceptionally high gliding speed. These caveats suggest that wind assistance would substantially increase the likelihood of a successful ocean crossing, and is further discussed below.

The three remaining questions concerned the availability and performance of favorable winds, and we obtained answers using wind trajectory analysis. We showed, answering the second question, that favorable winds are available to the globe skimmer during both autumn and spring migration, but they vary considerably in availability over time. Thirdly, migration simulations that successfully crossed the Indian Ocean were demonstrated to arrive most often in Somalia and Oman during the autumn, and northwest India during spring. And fourth, we

TABLE 1 | Four minimum distances across the Indian Ocean, based on starting points corresponding to the four departure sites used in the wind trajectory analyses of potential globe skimmer autumn and spring migration routes.

Distance ID	Distance (km)	Departure site	Arrival site, given the shortest crossing over the Indian Ocean
Autumn 1	938	Gwarka lighthouse, Gujarat, India	Ras Al Jinz, Oman
Autumn 2	1,744	Vasco da Gama, Goa, India	Ras Madrakah, Oman
Autumn 3	2,536	Malé, the Maldives	Kap Hafun, Somalia
Spring 1	2,430	Guardafui lighthouse, Somalia	Rameshwar, Maharashtra, India

Arrival sites are approximate locations given the shortest possible crossing of the Indian Ocean.

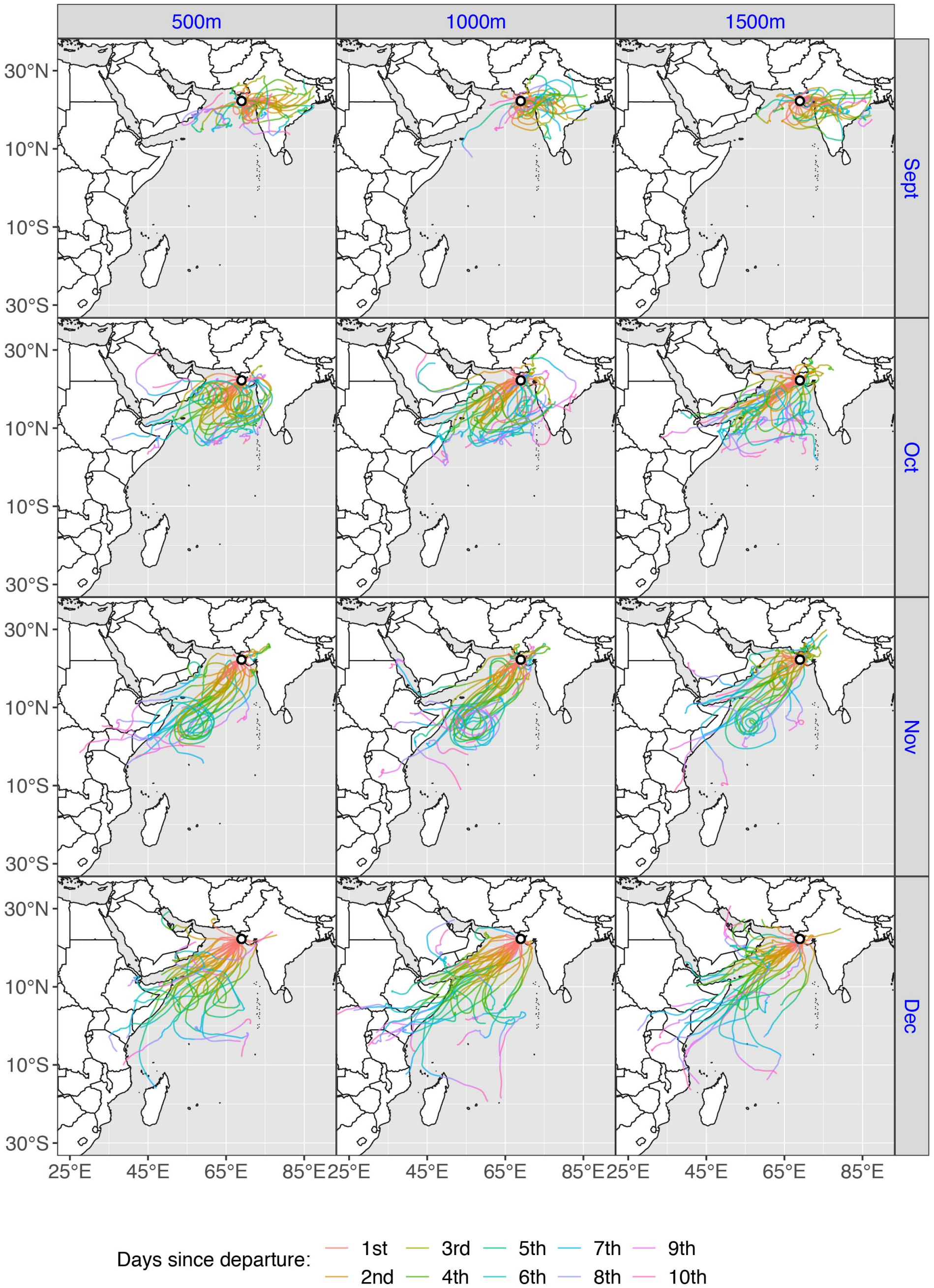

FIGURE 4 | Autumn wind trajectories (*N* = 366), during September–December, for three starting heights (500, 1,000, and 1,500 m) from the departure site Gwarka lighthouse, Gujarat, northern India. Colors denote the number of days since departure (1–10). As the model adheres to a limited geographical scope, trajectories that exceeded the boundary (approximately 87°E longitude) will stop, thus trajectories traveling eastward across the Bengal Sea are cut short.

find that a transoceanic migration assisted by winds would take an estimated average of 127 h in autumn but only 55 h in spring. Summarizing these findings, we show that variability in the availability of favorable winds supposes a requirement for wind selectivity on the dragonfly, if it is to successfully migrate across the Indian Ocean. Furthermore the simulated migration events have several interesting implications, which will be addressed in the following sections.

According to our estimates, active flight will deplete the energy reserves of a standard globe skimmer within just 4.39 h. This energy expenditure appears rapid when compared to exhaustion trials in other flying insects (Tigreros and Davidowitz, 2019). However, this figure represents a conservative estimate of flight duration based on the upper limits of metabolic rate (0.19 W). Similarly, our assumption that gliding metabolic rate is low and near that of resting (0.002904 W) should be

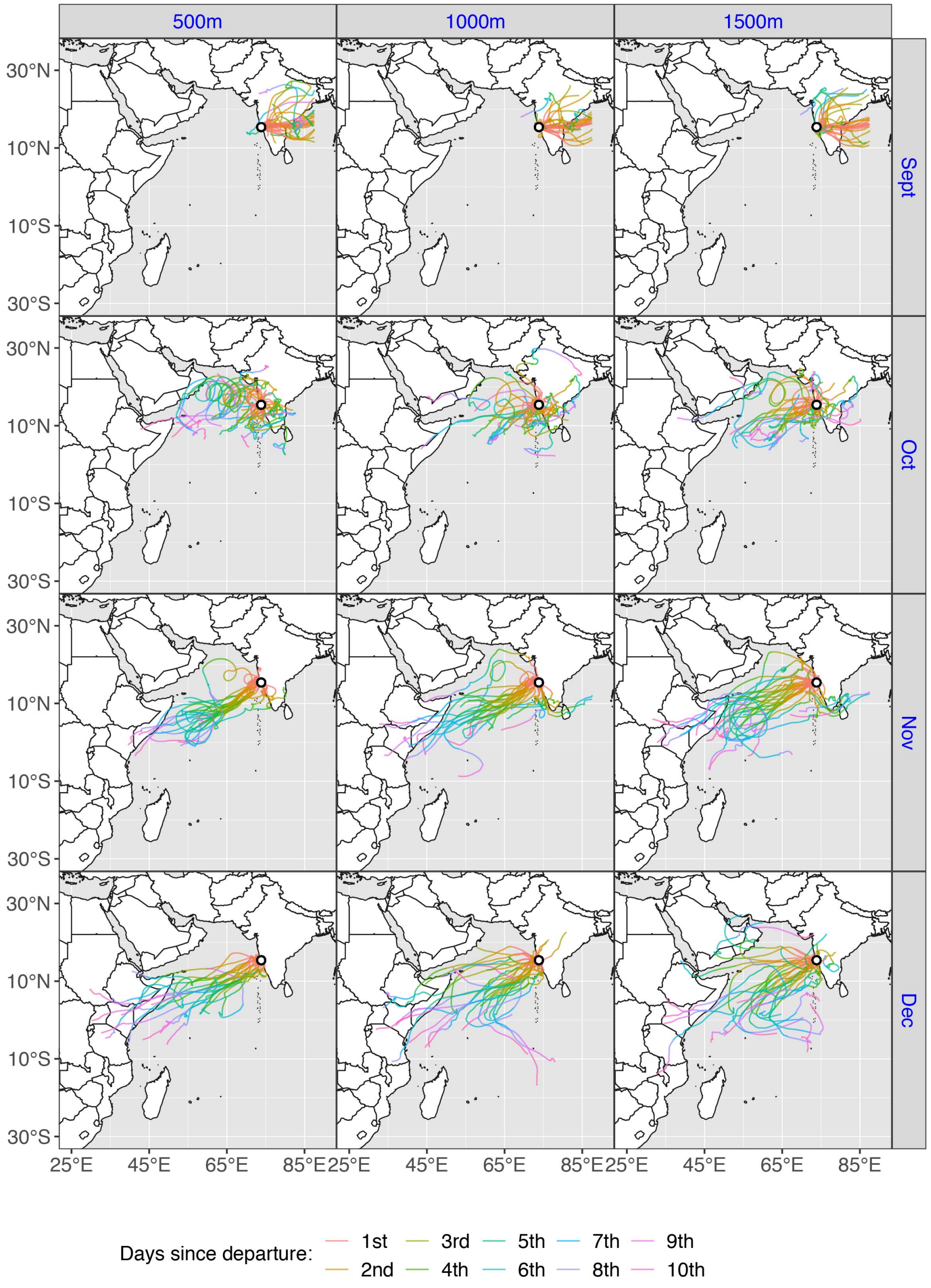

FIGURE 5 | Autumn wind trajectories (*N* = 366), during September–December, for three starting heights (500, 1,000, and 1,500 m) from the departure site Vasco da Gama, Goa, western India. Colors denote the number of days since departure (1–10). As the model adheres to a limited geographical scope, trajectories that exceeded the boundary (approximately 87°E longitude) will stop, thus trajectories traveling eastward across the Bengal Sea are cut short.

considered a minimum estimate. Thus, in reality, flight capacity likely lies somewhere within our upper and lower estimations. Nevertheless, with this consideration in mind we conclude that self-powered flight relying solely on a flight mode of continuous flapping flight, requiring high energy expenditure, will deplete reserves too quickly to allow the completion of a migratory journey across the Indian Ocean. Even if active flight is reduced to 20% and combined with a flight mode of low energy expenditure (i.e., gliding), this is unlikely to be sufficient to carry a globe skimmer across even the shortest distance crossing (i.e., Autumn 1), unless it experiences no headwind, and can operate at near its maximum metabolic rate constantly or at exceptionally high gliding speeds. Thus, we conclude that globe skimmer migration across the Indian Ocean would require wind assistance.

Our wind trajectory analyses clearly demonstrate that there are both intra- and inter-seasonal differences in favorable wind

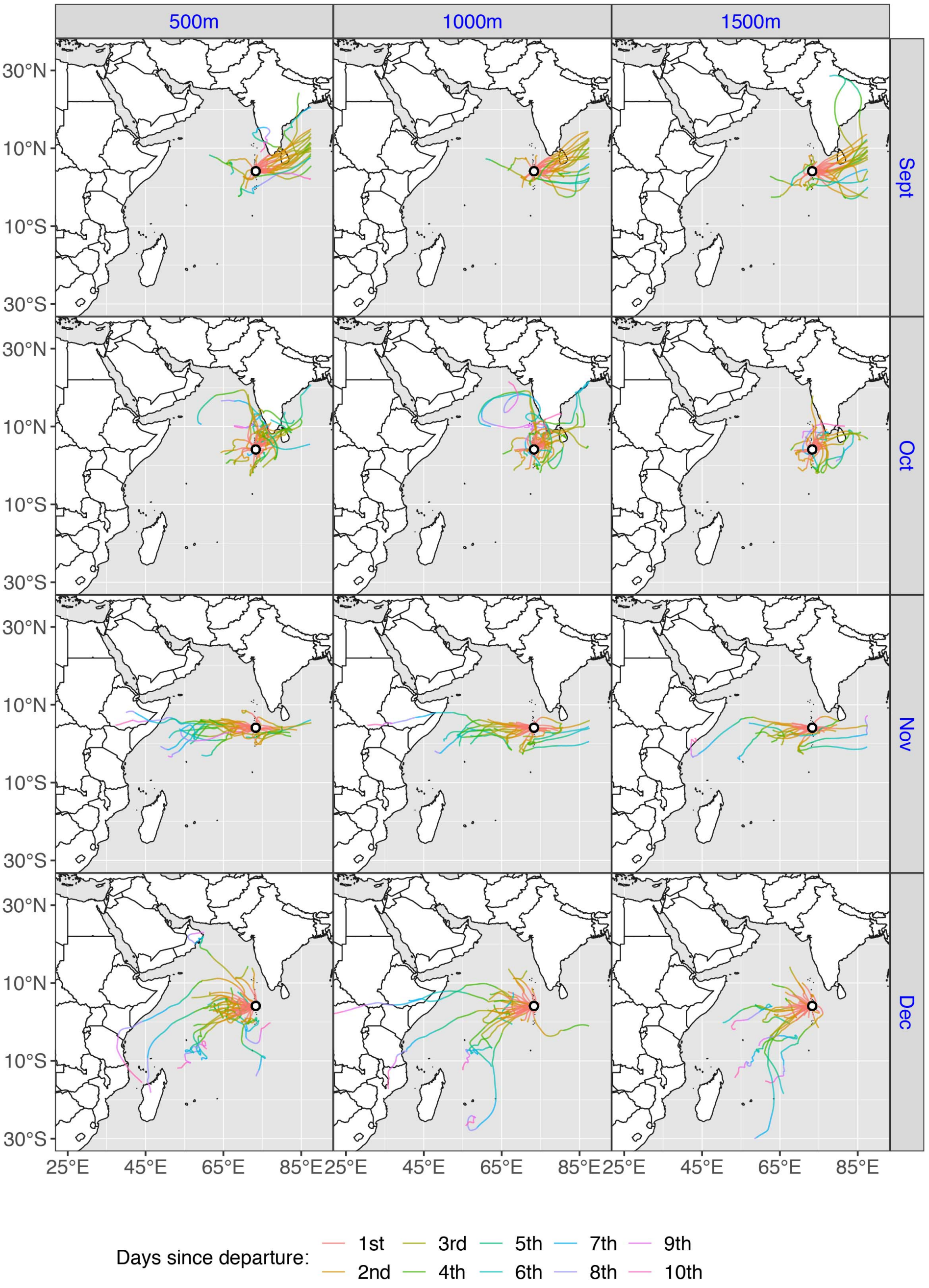

FIGURE 6 | Autumn wind trajectories (*N* = 366), during September–December, for three starting heights (500, 1,000, and 1,500 m) from the departure site Malé, the Maldives. Colors denote the number of days since departure (1–10). As the model adheres to a limited geographical scope, trajectories that exceeded the boundary (approximately 87°E longitude) will stop, thus trajectories traveling eastward across the Bengal Sea are cut short.

availability, suggesting that optimal, or even feasible, migration over the Indian Ocean requires the ability to select favorable winds. Indeed, only 15.2% of all simulated migration events in autumn were successful in reaching land, taking approximately 127 h to do so. Dragonfly migration is often reported to be associated with persistent winds, weather fronts and even storms (Corbet, 1962; Easton and Liang, 2000; Moskowitz et al., 2001; Srygley, 2003; Ries et al., 2018), and the globe skimmer is referred to as an obligate migrant of the ITCZ winds (Corbet, 2004). Indeed, there are numerous studies on insect migration suggesting that larger migrants (> 10 mg) have the ability to select winds, choosing to migrate in fast high-altitude airstreams, compensate for cross-wind drift, and select winds that will transport them in seasonally favorable directions (Chapman et al., 2008, 2010; Stefanescu et al., 2013; Wotton et al., 2019; Gao et al., 2020b). Compensating for cross-wind drift has

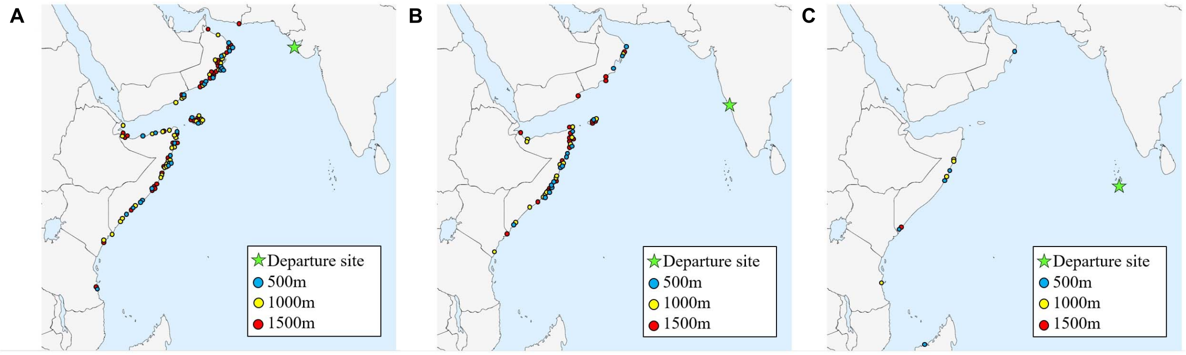

FIGURE 7 | Arrival sites for all successful autumn trajectories (*N* = 167), for each starting height (500, 1,000, and 1,500 m), and three departure sites: **(A)** Gwarka lighthouse, northern India (*N* = 105); **(B)** Vasco da Gama, western India (*N* = 53); and **(C)** Malé, the Maldives (*N* = 9).

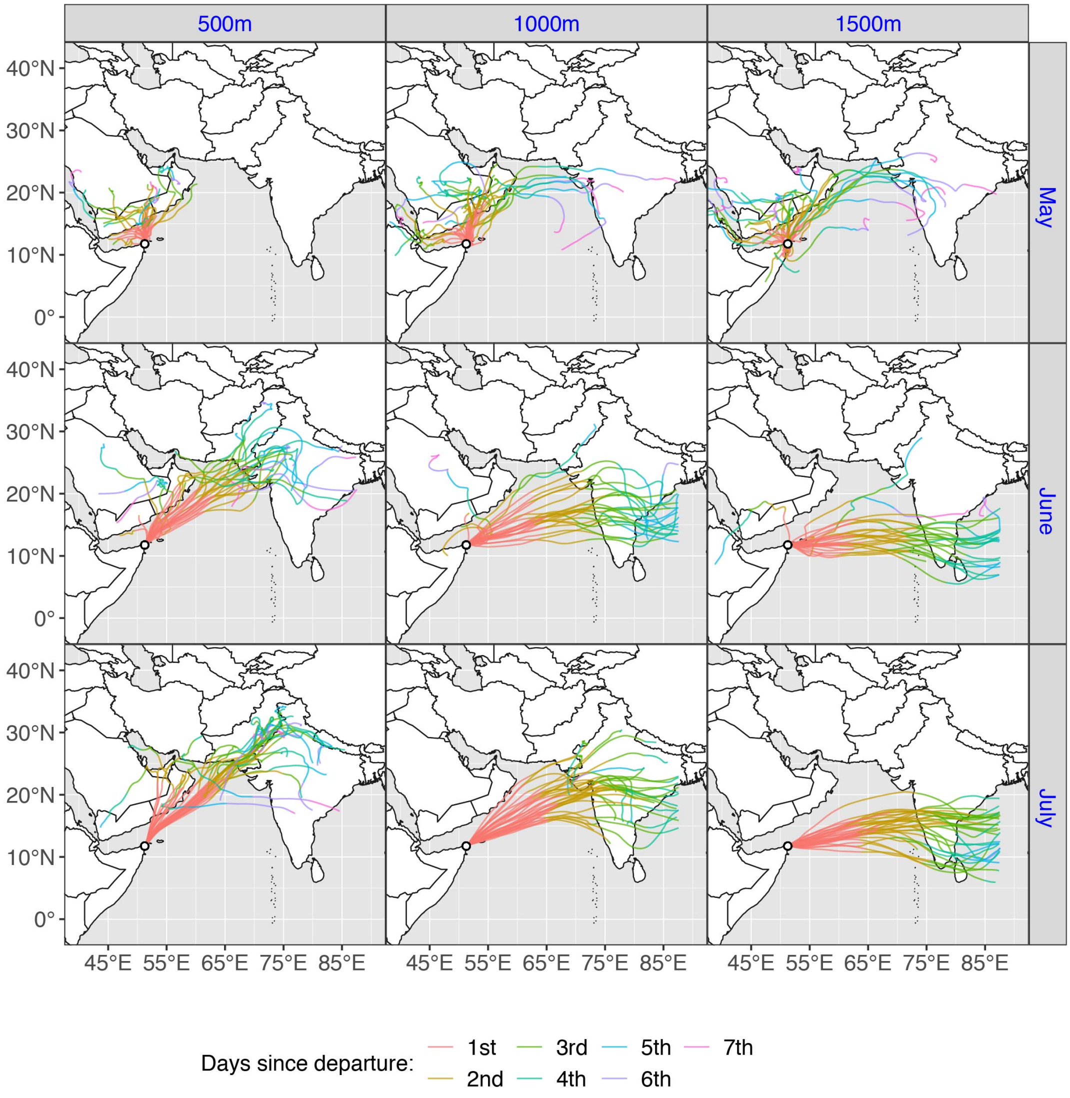

FIGURE 8 | Spring wind trajectories (*N* = 366) for one departure site, Guardafui lighthouse, Somalia, during May–July, for three starting heights (500, 1,000, and 1,500 m). Colors denote the number of days (flystage) since departure (1–6). As the model adheres to a limited geographical scope, trajectories that exceeded the boundary (approximately 87°E longitude) will stop, thus trajectories traveling eastward across the Bengal Sea are cut short.

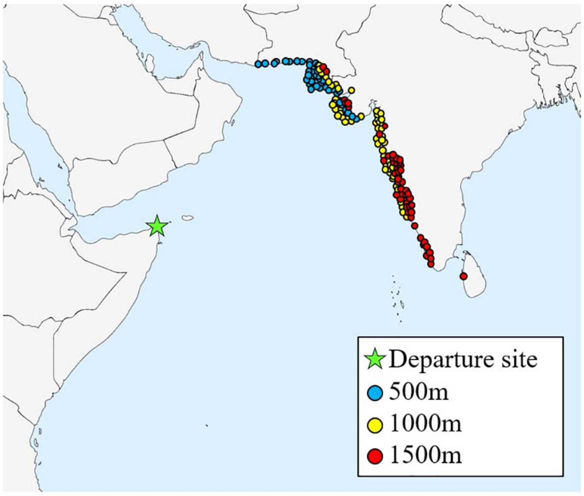

FIGURE 9 | Map showing arrival sites for all successful spring trajectories (*N* = 150), for each starting height (500, 1,000, and 1,500 m), for the departure site Guardafui lighthouse, Somalia.

been confirmed in the globe skimmer (Srygley, 2003), and it is likely that a species of this size, that is highly adapted for migration, would possess the ability to select winds. The ability and opportunity to select winds would be further increased if the dragonfly has physiological adaptations allowing it to remain airborne in unfavorable conditions, or when wind assistance is lacking. Consequently, investigating the mechanisms used by globe skimmers to select favorable winds for migration represents an interesting future research avenue.

According to our autumn wind trajectories in September, the month when reports of globe skimmer migration usually start in India (Fraser, 1924, 1954; Mitra, 1974, 1994), favorable winds across the Indian Ocean are almost completely absent, irrespective of departure site. These poor conditions prevail through October, at least for the two more southern departure sites, while the most favorable months to depart are November and December. In agreement with this finding, these later months coincide with the observed peak of globe skimmer passages across the Maldives (Anderson, 2009).

Within the African continent, globe skimmers appear in connection with seasonal rains and the movement of the ITCZ, in both northern and southern Africa (Corbet, 1962; Stortenbeker, 1967; Happold, 1968; Suhling and Martens, 2007; Corso et al., 2017). Unfortunately, associating the timing of the autumn wind trajectories with African observations is not possible, since there are no published observations of migrating globe skimmers arriving to, or departing from, East-African coastlines. There are, however, records from Indian Ocean islands situated east of the African coastline documenting the arrival of globe skimmers, for example in December in the Seychelles (Campion, 1913) and in October–November in the Chagos Archipelago (Carr, 2020).

The regular appearance of thousands of dragonflies on the Maldives brings in to question why so few trajectories (9 out of 366) were successful in their simulated migration from the Maldives. If our simulations were truly representative of the migratory effort, that would imply very high mortality of globe skimmers transiting the Maldives. Such prodigal waste of life, as assumed for example by Fraser (1954), may be an inevitable consequence of an obligate migratory lifestyle governed by the ITCZ (Corbet, 2004) or by migration in general (Sillett and Holmes, 2002). The globe skimmer may benefit from moving on seasonal winds for migration throughout most of its range (e.g., over mainland continents), but could face a higher likelihood of failure in certain areas (e.g., over the Pacific and Indian Ocean). In fact, stable isotope analysis of individuals arriving on the Maldives indicate that their origin is northern India (Hobson et al., 2012), and having already traveled > 2,000 km, they could be at the limit of their migratory effort.

The low success rate of trajectories starting in the Maldives may also reflect model inadequacy. Our simulations are (mainly)

TABLE 2 | Successful autumn and spring simulated migrations events from the wind trajectory analysis.

Season	Departure site	Departure height	Number of trajectories	Flight time (average, h)	Flight time (shortest, h)	Flight time (longest, h)
Autumn	Gwarka lighthouse	500	28	119.25 ± 9.4	33	233
Autumn	Gwarka lighthouse	1,000	38	112.07 ± 7.1	51	230
Autumn	Gwarka lighthouse	1,500	39	112.66 ± 7.7	39	212
Autumn	Vasco da Gama	500	16	152.3 ± 11.2	71	232
Autumn	Vasco da Gama	1,000	17	153 ± 13.1	68	212
Autumn	Vasco da Gama	1,500	20	137.2 ± 10.5	68	232
Autumn	The Maldives	500	4	173.5 ± 11.7	140	194
Autumn	The Maldives	1,000	4	144 ± 12.8	127	182
Autumn	The Maldives	1,500	1	208	208	208
Spring	Somalia	500	42	62.5 ± 2.2	34	96
Spring	Somalia	1,000	56	69.3 ± 2.9	33	141
Spring	Somalia	1,500	52	87.6 ± 3.1	32	146

The table lists the number of successful trajectories for each departure site and starting height, the average flight time and the shortest and longest flight time for each departure site and height.

based on wind data from 2019, which was an atypical year, with regional oceanography and meteorology dominated by a positive phase of the Indian Ocean Dipole (Ratna et al., 2021). Furthermore, the wind trajectory model assumed migration downwind, but depending on the extent of globe skimmer wind selection ability, it could optimize wind usage further, for example by actively seeking out and exploiting rising air (Gibo, 1981) thus enabling faster and longer journeys. For example, great frigatebirds (*Fregata minor*) may stay aloft for months over the Indian Ocean using thermal lift, carefully selecting favorable winds and tracking the convections of the ITCZ (Weimerskirch et al., 2016). Thus, as most dragonflies leave the Maldives, it is possible that the archipelago functions as a stop-over and refueling site, and that many transit migrants do in fact make it across the ocean.

On the Arabian Peninsula, globe skimmer migratory movements are noted annually and regularly in October and November (Feulner, 2003; Campbell, 2009; Porter, 2011), including observations of large swarms occurring along the coast of UAE, the Island of Socotra (Yemen), and Oman (Campbell, 2009; Porter, 2011; Lambret et al., 2017). Successful wind trajectories departing from Gwarka lighthouse, northern India, did reach land in this region, resembling, in part, a proposed migration scenario suggested by Borisov et al. (2020b). But whether this confirms that Indian globe skimmer migrants do cross the Indian Ocean aiming for the Arabian Peninsula remains speculative until more detailed field studies are carried out. Nevertheless, our results identify this as a feasible migratory route for the species, in addition to routes further south directly across the Indian Ocean or via the Maldives.

In spring, wind trajectories demonstrate a much faster transoceanic crossing than in autumn, carrying a globe skimmer across the Indian Ocean within ∼55 h during May–August. This period follows the globe skimmer's second flight period in The Arabian Peninsula (March–April) (Waterston and Pittaway, 1991), suggesting this area as a possible origin of migrants departing for India. The monsoonal rains return to India in May, making the area suitable for globe skimmer reproduction again (Corbet, 1988). Reappearances of the globe skimmer in India following these rains are sometimes described as "sudden" (Corbet, 1988), and considering the highly favorable winds available to transport migrants from eastern Africa at this time, it is possible that at least some of the individuals that suddenly appear in India are transoceanic migrants (Anderson, 2009). An alternative origin is wetter areas in Sri Lanka and within India itself, for example the states of Kerala and Tamil Nadu, where globe skimmers are on the wing year-around (Corbet, 1988; Muthukumaravel et al., 2015).

A third potential source of globe skimmers appearing in India in May–July is the vast areas of rice paddy fields in Southeast Asia. For comparison, recent research on the brown planthopper (*Nilaparvata lugens*) has revealed substantial exchange between populations from India, Southeast Asia and temperate East Asia (Hu et al., 2019; Gao et al., 2020a). Similar migratory routes and population exchange could take place in the globe skimmer. Indeed, the seasonal globe skimmer migrations through China, Russia, Southeast Asia and Japan, already suggest substantial population mixing throughout Asia (Gibson-Hill, 1950; Feng et al., 2006; Borisov and Malikova, 2019; Hobson et al., 2021), and the origin of globe skimmers arriving on the Maldives has been traced to NE India or beyond (Hobson et al., 2012). Mapping the migratory movements of globe skimmers within Asia, and between Asia and other continents, thus presents an excellent framework for the study of large-scale migration in a common and ecologically influential, but non-pest, insect.

Besides the globe skimmer, several bird species are known or presumed to migrate over the Indian Ocean (Gaston, 1976; British Trust For Ornithology, 2020), including the Amur falcon *Falco amurensis* (Dixon et al., 2011; The Amur Falcon Partnership, 2016; Meyburg et al., 2017). Comparing actual GPS-tracked routes of Amur falcons (Dixon et al., 2011; The Amur Falcon Partnership, 2016), with our autumn wind trajectories departing from Vasco da Gama (Goa), the routes correspond extremely well. Similarly, in spring, Amur falcon departure appears to hug the coastline of Somalia, with birds arriving into north India (The Amur Falcon Partnership, 2016; Meyburg et al., 2017), in clear agreement with our spring wind trajectories, especially the 500 m simulations. These similarities suggest the possibility that the Amur falcon co-migrates with the globe skimmer, selects similar, favorable winds, and potentially even utilizes migrating dragonflies as a prey resource during the crossing (Anderson, 2009).

Our results for aerial migration over the Indian Ocean reinforce the importance of seasonally favorable winds, and that a whole flyway system, involving several trophic levels, may be susceptible to disruption if wind patterns alter. Models of climatic change have indicated that the positioning of the ITCZ, and the behavior of the trade winds, can shift with pronounced temperature change within timescales as short as decades (Broccoli et al., 2006; Green et al., 2017). The implications of pronounced wind system change are currently unclear. However, there is a potential to disrupt migratory flyways and dislodge ecological exchange between and within ecosystems, emphasizing the importance of further research into the dynamics of wind-assisted migratory dynamics.

To conclude, our simulations estimate the circumstances whereby globe skimmers can successfully migrate over the Indian Ocean. In the few occasions where field observations are available, these correspond well with our wind trajectories. Thus, the open question "do dragonflies migrate over the western Indian Ocean?" (Anderson, 2009), can receive a cautious confirmation from a physiological and meteorological perspective. However, there is still several aspects of this proposed bidirectional transoceanic migration in need of verification: i.e., confirmed arrival of Indian migrants to reproductive habitats across the Indian Ocean, and the departure and arrival of a subsequent generation back to India. Expanding insect radar monitoring in Africa, as well as in India, is one suggested future research aim that would greatly increase our knowledge on the movement patterns of this and other important species. Another interesting avenue to explore would be life-cycle modeling, where benefits and costs of migration could be studied through population dynamics, as has been previously done for avian migrants. As the globe skimmer is a common and highly migratory species with a worldwide distribution, it is a promising model system for movement ecology research, and the Indian Ocean migration is just one of its many noteworthy flyways worth detailed investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JH, JC, and RCA developed the concept and scope, as well as main text of this study. HL, PL, and GH developed and executed the modeling. JH interpreted the model results. JH, HL, and PL designed and produced the figures and tables. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.698128/full#supplementary-material

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Snow Buntings Maintain Winter-Level Cold Endurance While Migrating to the High Arctic

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Arctic breeding songbirds migrate early in the spring and can face winter environments requiring cold endurance throughout their journey. One such species, the snow bunting (*Plectrophenax nivalis*), is known for its significant thermogenic capacity. Empirical studies suggest that buntings can indeed maintain winter cold acclimatization into the migratory and breeding phenotypes when kept captive on their wintering grounds. This capacity could be advantageous not only for migrating in a cold environment, but also for facing unpredictable Arctic weather on arrival and during preparation for breeding. However, migration also typically leads to declines in the sizes of several body components linked to metabolic performance. As such, buntings could also experience some loss of cold endurance as they migrate. Here, we aimed to determine whether free-living snow buntings maintain a cold acclimatized phenotype during spring migration. Using a multi-year dataset, we compared body composition (body mass, fat stores, and pectoralis muscle thickness), oxygen carrying capacity (hematocrit) and metabolic performance (thermogenic capacity – M_{sum} and maintenance energy expenditure – BMR) of birds captured on their wintering grounds (January–February, Rimouski, QC, 48°N) and during pre-breeding (April–May) in the Arctic (Alert, NU, 82°). Our results show that body mass, fat stores and M_{sum} were similar between the two stages, while hematocrit and pectoralis muscle thickness were lower in pre-breeding birds than in wintering individuals. These results suggest that although tissue degradation during migration may affect flight muscle size, buntings are able to maintain cold endurance (i.e., M_{sum}) up to their Arctic breeding grounds. However, BMR was higher during pre-breeding than during winter, suggesting higher maintenance costs in the Arctic.

Keywords: Arctic bird, Arctic breeding, body composition, basal metabolic rate, cold acclimatization, migration, phenotypic flexibility, summit metabolic rate

INTRODUCTION

Arctic breeding birds typically migrate early in the spring to maximize the chances of successful reproduction during a relatively short summer (Love et al., 2010; Reneerkens et al., 2016; van Gils et al., 2016; Ramenofsky and Wingfield, 2017; Rakhimberdiev et al., 2018). For these species, early arrival allows for securing the best breeding territories and partners, and for breeding as soon as conditions become suitable (Drent et al., 2003; Ramenofsky and Wingfield, 2006), which can improve reproductive success (Lepage et al., 2000; Guindre-Parker et al., 2013). However, such early arrival implies migrating through cold winter conditions and, upon arrival on Arctic breeding grounds, birds are often faced with substantial snow accumulation, sub-zero temperatures and unpredictable weather that can persist for several weeks (e.g., Meltofte, 1983; Walsh et al., 2005; see also Morrison et al., 2007; Wingfield et al., 2011).

For species that overwinter in cold environments (e.g., temperate zones), these arrival conditions may be comparable or even more constraining than those encountered during winter (Meltofte, 1983; Wingfield et al., 2011; Snell et al., 2018). However, the physiological mechanisms involved in acclimatization to cold and to long-distance migration share similar physiology (Dawson et al., 1983; Zhang et al., 2015). For example, heart mass, blood oxygen-carrying capacity (hematocrit), and flight muscle size increase during cold acclimatization (Swanson, 1990b; O'Connor, 1995, 1996; Cooper, 2002; Zheng et al., 2008; Liknes and Swanson, 2011; Petit et al., 2013, 2014; Swanson and Vézina, 2015), and are thought to improve shivering heat production (Swanson, 1990a; Petit and Vézina, 2014). Similar changes also occur in preparation for migration (Battley and Piersma, 1997; Piersma et al., 1999; Krause et al., 2016) to support active flight (Battley and Piersma, 1997; Piersma et al., 1999). Therefore, birds migrating early in the spring could profit from a carryover of their winter phenotype to benefit migration in the cold.

Recently, Le Pogam et al. (2021) observed that snow buntings (*Plectrophenax nivalis*), Arctic breeding songbirds known for their cold endurance (Scholander et al., 1950; Le Pogam et al., 2020), were able to maintain a winter phenotype through most of summer when kept in outdoor aviaries at their wintering latitude (48°N), at a time when air temperatures can exceed 25°C. More precisely, the birds were found to maintain thermogenic capacity and cold endurance comparable to those from the peak of winter not only throughout migratory fattening, but also during the period corresponding to migration and Arctic breeding. Le Pogam et al. (2021) concluded that the winter phenotype in snow buntings transitioning into spring migration provided enough thermogenic capacity to migrate in cold, winter-like environments and to support thermoregulatory needs upon arrival on their breeding grounds. However, that study was based on captive individuals that could not migrate, and migration also has considerable physiological effects, such as the loss of body mass as well as declines in oxygen carrying capacity and muscle mass, which together could both potentially lead to a reduction in thermogenic capacity (O'Connor, 1995; Cooper, 2002; Jenni et al., 2006; Dubois et al., 2016). Thus, whether this cold specialist maintains winter-level cold endurance through spring migration remains to be tested.

The objective of this study was to compare peak of winter and pre-breeding phenotypes in free-living snow buntings to determine whether these birds show comparable metabolic performance and supporting traits at those stages, which would lend strength to the hypothesis that buntings maintain cold endurance throughout their spring migration (as proposed by Le Pogam et al., 2021). To test this hypothesis we compared phenotypic traits related to thermogenic capacity (**Table 1**) in buntings captured around Rimouski, Québec, Canada (48°N) during the peak of winter (i.e., January and February) to those of individuals captured in the weeks following arrival (i.e., May and early June) on breeding grounds at Alert, NU, Canada (82°N). Based on our previous observations (Le Pogam et al., 2021), we expected buntings would maintain a winter-type phenotype (**Table 1**) throughout both migration and pre-breeding. Birds would thus be expected to have comparable metabolic performance during winter in Québec and early spring in the Arctic. In contrast, if migration leads to body transformations that negatively impact metabolic performance at subsequent stages, we would instead expect to find lighter birds in the spring showing lower performance levels compared to individuals measured during winter.

MATERIALS AND METHODS

Study Species

Snow buntings are an Arctic-breeding, cold-associated migratory passerine. In the spring, these birds migrate through cold winter landscapes (Macdonald et al., 2012; McKinnon et al., 2016; Snell et al., 2018), with males arriving on the breeding grounds to secure territories (up to 83.6°N) up to a month before females (March–April, Cramp and Perrins, 1994; McKinnon et al., 2016; Snell et al., 2018; Montgomerie and Lyon, 2020). Arrival conditions can be comparable or even harsher than those experienced at the peak of winter, with extensive snow cover and air temperatures (T_a) reaching −30°C (Meltofte, 1983). After arrival, buntings can maintain winter-like behavior for several weeks before dispersing to defend breeding territories (Tinbergen, 1939; Meltofte, 1983). Although the wintering range of birds breeding at Alert has yet to be formally established as no banded individuals have been recovered so far, banding data analyzed by Meltofte (1983; Figure 1) and Macdonald et al. (2012) suggest that Alert birds could be wintering in either North America or in the Siberian steppes. Regardless, in both cases wintering conditions are similar to that experienced by birds in Eastern Québec. For example, Snell et al. (2018) tracked snow buntings breeding at Svalbard and reported a mean temperature on their wintering range (i.e., Siberian steppes) of −10.9°C to −3.6°C, which encompasses the mean temperature on the wintering ground for this study (−8.9°C in February, **Table 2**).

Ethics Statement

All bird handling at Rimouski (QC) and at Alert (NU) was approved by the Animal Care Committee of the Université du

TABLE 1 | List of phenotypic traits measured in this study and their responses to cold and winter in passerine birds.

Metabolic performance

Phenotypic traits	Interpreted as	Response to cold/winter	Pertinent references
Summit metabolic rate (M_{sum})	Maximum shivering thermogenic capacity, index of cold endurance	Typically higher	McKechnie and Swanson, 2010; Swanson, 2010; Petit et al., 2013; McKechnie et al., 2015; Le Pogam et al., 2020
Basal metabolic rate (BMR)	Maintenance energy expenditure, index of physiological maintenance costs	Often higher but not in snow buntings	McKechnie and Swanson, 2010; Swanson, 2010; Petit et al., 2013; McKechnie et al., 2015 but see Le Pogam et al., 2020

Phenotypic traits underlying metabolic performance

Phenotypic traits	Interpreted as	Response to cold/winter	Pertinent references
Body mass	Total body composition	Typically higher	Carey et al., 1978; Liknes and Swanson, 1996; Zheng et al., 2008; Petit et al., 2014; Le Pogam et al., 2020
Fat store	Energy reserves	Typically higher	Blem, 1976; Lehikoinen, 1987; Gosler, 1996; Cooper, 2007; Le Pogam et al., 2020
Hematocrit	Blood oxygen carrying capacity	Typically higher	Swanson, 1990b; O'Connor, 1996; Le Pogam et al., 2020
Pectoralis muscle thickness	Shivering capacity	Typically increases with high metabolic rate	O'Connor, 1995; Cooper, 2002; Swanson and Merkord, 2012; Petit et al., 2013; Swanson and Vézina, 2015; Le Pogam et al., 2020

Québec à Rimouski (Rimouski: CPA-54-13-130 and CPA-71-17-195 and Alert: CPA-61-15-163 and CPA-71-17-194) and was conducted under banding (10889E) and scientific (SC-48, NUN-SCI-15-05) permits from Environment and Climate Change Canada, and under scientific permits from the Department of Environment of Nunavut (WL 2016-006, 2017-021, 2018-010, 2019-002).

Study Sites, Capture, and Measurements Protocol

On the Breeding Grounds

Snow buntings were studied in the Arctic during the springs of 2016–2019 at Alert, NU, Canada (82°29′58″N, 62°28′5″W). Specifically, we studied birds from their arrival to their dispersal onto breeding territories (hereafter pre-breeding, $n = 213$ males and 53 females, see **Supplementary Table 1** for detailed sample sizes per variable). Although single individuals or small groups (i.e., 2–4 birds) can be observed earlier, the bulk of arrivals at Alert occur in the last week of May (AL, FV, pers. obs.).

Birds were caught with homemade walk-in traps or potter traps (Third Wheel, Devon, England) baited with commercial seed-mix (crushed corn, wheat, sorghum, white millet, red millet, and black sunflower, Armstrong, Hagersville, ON, Canada).

Immediately after capture, a blood sample (<1% of body mass) was taken from the brachial vein. Blood samples were temporarily kept in cold storage and later centrifuged for 10 min at 8,000 RPM to obtain data on hematocrit (i.e., packed red blood cell volume). The birds were then weighed (±0.01 g) and sexed according to Smith (1992). We banded birds with a USGS numbered metal band as well as a unique combination of three darvic color bands to allow for individual identification from a distance. Right wing length was measured as an index of structural body size. The size of fat stores was also estimated visually using a standard fat score (from 0 = no visible fat in furculum area to 6 = fat overlapping pectoralis muscles according to Canadian Snow Bunting Network guidelines, Love et al., 2012). The birds were then brought into our field laboratory (less than 6 km distance from capture site, transport time <20 min) where we estimated pectoralis muscle thickness non-invasively by ultrasonography (Dietz et al., 1999; Royer-Boutin et al., 2015; Le Pogam et al., 2020, 2021) using a LOGIQ e ultrasound scanner fitted with a linear probe (12 MHz, GE Healthcare, Wauwatosa, WI, United States). Since the supracoracoideus muscle is very thin at the measured location, muscle thickness values essentially reflect thickness of the pectoralis muscle. Birds were then held in cages (76 cm W × 46 cm D × 45 cm H) with *ad libitum* water and seed (same mix as for captures) until metabolic performance measurements were complete (see below).

On the Wintering Grounds

Each winter between January and February from 2015 to 2018, wintering buntings were captured around Rimouski, QC, Canada (48°27′N, 68°30′W) as part of a snow bunting banding program. Snow buntings have a differential migration (Macdonald et al., 2016; McKinnon et al., 2019) and the wintering population around Rimouski is composed in very large proportion of males (total captures males = 508, females = 31, see **Supplementary Table 1** for detailed sample sizes per variable). Birds were captured using walk-in traps baited with crushed corn. Upon capture, birds were subjected to the same measurement sequence as described above except that they were banded with only a USGS numbered metal band. Blood samples were taken on 51 individuals and later centrifuged for 10 min at 8,000 RPM (UNICO PowerSpin BX Centrifuge C886, Dayton, NJ, United States) to obtain hematocrit data. In total, 57 birds were transported to the avian facilities at the Université du Québec à Rimouski (less than 25 km distance from the capture site, transport time <25 min) for pectoralis muscle thickness and metabolic performance measurements. Birds were held in indoor cages (117 cm W × 310 cm D × 39 cm H) with access to *ad libitum* food and water while waiting for measurements.

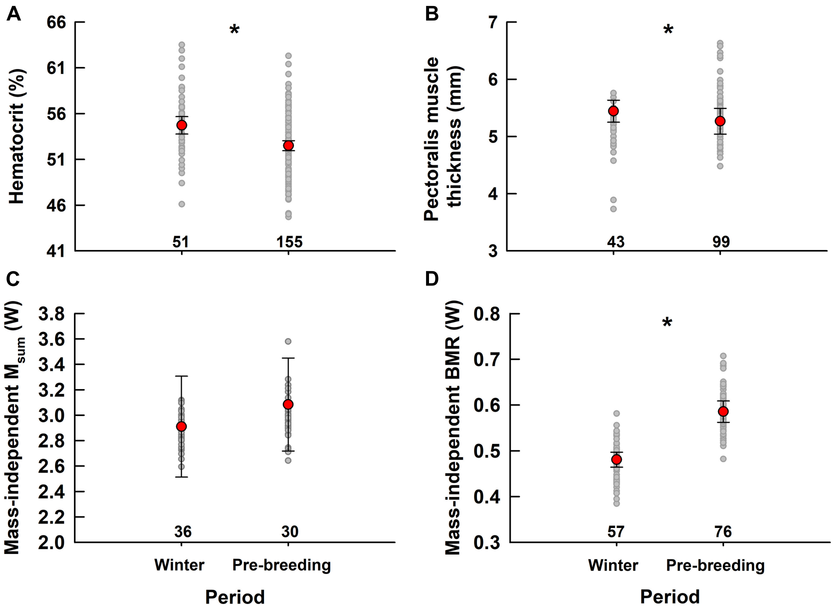

FIGURE 1 | Comparing hematocrit **(A)**, pectoralis muscle thickness **(B)**, mass-independent M_{sum} **(C)** and mass-independent BMR **(D)** in snow buntings measured in winter in Rimouski, Québec, Canada and during pre-breeding at Alert, NU, Canada. A star indicates significant differences. Numbers indicate sample size for each group. Values in red are least square means ± CI controlling for the random effect of year and for covariates in models including body mass for M_{sum} and BMR, thus making these variables "mass-independent." Gray dots show data predicted by models including fixed and random effects.

Metabolic Performance

For both Alert and Rimouski, we used the set-up and protocol described by Le Pogam et al. (2020, 2021), except that the oxygen analyzers used at Alert were two Sable Systems Foxboxes (Sable Systems, Las Vegas, NV, United States) instead of the Servomex oxygen analyzer (gas purity analyzer, model 4100, Boston, MA, United States) used at Rimouski. The following key points are specific to this study.

Summit metabolic rate (M_{sum} – the maximum metabolic rate in response to the cold, considered a measure of cold endurance; Dutenhoffer and Swanson, 1996) was measured on up to two birds simultaneously, allowing for two trials per day. Depending on the time of capture, measurements began between 10:04 and 22:45 (average: 15:09 ± 3.27 h; duration: 1.68 ± 0.89 h) at Alert, and between 10:14 and 17:10 (average: 12:26 ± 2.05 h; duration: 1.88 ± 0.67 h) at Rimouski. Measurements took place at least 1 h after ultrasound measurements. Birds were placed inside stainless steel metabolic chambers (effective volume 1.5 L) and exposed to dry, CO_2-free air for 10 min at −18°C (flow rate of 1,200 mL.min^{-1}), before switching to a helox gas mixture (21% oxygen, 79% helium, Rosenmann and Morrison, 1974). Chamber temperature was then lowered by 3°C every 20 min until birds became hypothermic [decline of birds' oxygen consumption ($\dot{V}O_2$) for several minutes] or reached the end of the trial programmed time (125 min). At Alert, 5 measurements out of 30 (16.6%) involved birds that were not hypothermic at the end of their trial (cloacal temperature ≥37°C, Swanson and Liknes, 2006). There were no such cases at Rimouski. However, since hypothermia is not a prerequisite to confirm M_{sum} (Dutenhoffer and Swanson, 1996), we included these five individuals in our analyses (removing them did not affect results). Basal metabolic rates (BMR – metabolic rate at rest considered a measure of physiological maintenance; McNab, 1997; Swanson et al., 2017) were measured overnight on a maximum of four birds simultaneously. At Alert, BMR trials began between 18:56 and 0:22 (average start time: 21:12 ± 1.49 h) and at Rimouski between 17:03 and 21:19 (average start time: 18:58 ± 1.27 h). Since M_{sum} and BMR were assessed consecutively, we ensured a minimum 1 h of rest after the M_{sum} measurements. Using the same metabolic chambers as for M_{sum}, birds were exposed to 25°C, a temperature within snow bunting's thermoneutral zone (Scholander et al., 1950) and received dry CO_2-free air (650 ml min^{-1}) for the duration of trials (10.88 ± 1.81 h on average at Alert, 13.49 ± 1.84 h at Rimouski). Birds were weighed (±0.01 g) before and after measurements, and average body mass (M_b) was used in BMR analyses. We used a sampling frequency of 5 s for M_{sum} and 20 s for BMR. Both M_{sum} and BMR were calculated from the highest and lowest averaged 10 min trace of $\dot{V}O_2$, respectively, using equation 10.1 from Lighton (2019) and using the instantaneous measurement technique (Bartholomew et al., 1981) for M_{sum}. The duration of BMR trials ensured that birds were post-absorptive at the time of BMR measurements. We estimated energy expenditure for all metabolic measurements using a constant equivalent of 19.8 kJ L$^{-1}O_2$ and converted units to Watts (Gessaman and Nagy, 1988).

Weather Data

Weather data for both field sites were obtained from nearby weather stations. For Rimouski, we used data from Pointe-au-Père station (48°30′50′′N; 68°28′06′′W, Government

TABLE 2 | Air temperatures recorded at the peak of winter at Rimouski (QC) averaged from 2015 to 2018 and in the spring at Alert (NU) averaged from 2016 to 2019.

	Ambient air temperature (°C)		
	Min	**Mean**	**Max**
Winter (Rimouski, QC)			
January	−12.5 ± 5.7	−8.6 ± 5.7	−4.0 ± 6.6
February	−13.2 ± 6.6	−8.9 ± 6.4	−4.3 ± 6.8
Spring (Alert, NU)			
April	−26.3 ± 4.7	−23.6 ± 4.6	−20.9 ± 5.0
May	−12.6 ± 4.8	−10.7 ± 4.6	−8.8 ± 4.7

of Canada)[1]. For Alert, we used data collected by Environment and Climate Change Canada at their Alert weather station (i.e., our study site).

Statistical Analyses

Our objective was to determine whether snow buntings retain a winter phenotype up to their pre-breeding stage. Given the known differences between sexes in phenotypic adjustments for breeding and wintering (see below), we also considered potential differences in patterns between sexes for body mass and fat score (not enough data in females for the other variables in winter). We therefore used linear mixed-effect models with M_b, fat score, hematocrit, pectoralis muscle thickness, M_{sum} and BMR as separate response variables. All models included "period" (i.e., winter or pre-breeding) as categorial predictor variables. The variable "year" was treated as a random parameter because we did not have measurements for all years in the two periods.

For M_b and fat score, we had sufficient data to consider the potential (fixed) effect of sex and the interaction "sex × period" in models. This is pertinent as mass and fat load could differ between sexes (Laplante et al., 2019). We also considered a potential effect of daily fattening on M_b and fat (e.g., Laplante et al., 2019) by including a covariate "time at capture." Analyses on M_b further considered the potential influence of structural body size by including wing length in models.

For pectoralis muscle thickness, "keel height" measured by ultrasound was included as a covariate to control for variation in muscle thickness due to probe positioning (Le Pogam et al., 2020, 2021).

We first analyzed metabolic performance parameters considering whole-animal M_{sum} and BMR. We then included M_b as a covariate in models to examine "mass-independent" variation. Under scenarios when whole and mass-independent results were similar, mass-independent values are presented. Since the birds could be caught at any time of the day, M_{sum} and BMR measurements could not always be conducted on the day of capture. Therefore, models also included "length of captivity."

Visual inspection of residuals confirmed assumptions of normality and homogeneity of residuals for all models. All analyses were conducted using JMP pro (14.0.0) and data are presented as mean ± standard error of the mean (SEM) in the text and 95% confidence intervals (CI) in graphs. Effects were considered significant and retained in models when $P < 0.05$. However, for one model, the interaction was marginally non-significant at $P = 0.06$. We therefore opted to keep this interaction term in the final model.

RESULTS

Weather Conditions

Air temperatures were lower at Alert during pre-breeding (April and May, **Table 2**) than at Rimouski during the coldest period of the winter (January and February, **Table 2**).

Phenotype Comparisons Between Winter and Pre-breeding

After considering the significant effects of body size and time at capture, M_b and fat score were comparable between winter and pre-breeding (M_b: winter = 38.8 ± 0.5 g, pre-breeding = 38.2 ± 0.5 g; fat score: winter = 3.3 ± 0.15 units, pre-breeding = 3.4 ± 0.15 units) despite a non-significant trend for birds being slightly heavier in winter (1.5%, **Table 3**). A significant sex effect was observed for M_b (**Table 3**), with males (39.6 ± 0.4 g) being heavier than females (37.4 ± 0.6 g) for their size. There was also a tendency for females to have higher fat reserves on the breeding ground while males remained unchanged (**Table 3**), but since there was a large overlap in data, this trend (not shown) was very weak. Hematocrit was lower during pre-breeding (52.5 ± 0.5%) than in winter (54.6 ± 0.6%; **Table 3** and **Figure 1A**). Pectoralis muscle thickness differed between periods (**Table 3**) with values being 3.12% higher during winter (5.44 ± 0.06 mm) than pre-breeding (5.27 ± 0.04 mm, **Figure 1B**).

Summit metabolic rate (M_{sum}), whether considered whole (not shown) or corrected for M_b, remained stable between winter and pre-breeding (**Table 3** and **Figure 1C**, winter = 2.9 ± 0.1W, pre-breeding = 3.1 ± 0.1W). After considering the significant effect of captivity duration (BMR decreases with captivity duration, data not shown), BMR, whole (not shown) or corrected for M_b, was 22.9% higher during pre-breeding (0.59 ± 0.01W) than in winter (0.48 ± 0.01W, **Table 3** and **Figure 1D**).

DISCUSSION

This study reports the northernmost measurements of thermogenic capacity ever measured in birds (i.e., Alert, NU, Canada – only 817 km from North Pole). We aimed to determine whether pre-breeding snow buntings expressed a phenotype comparable to that observed while wintering at lower latitudes, which would support the hypothesis that these birds maintain winter-level metabolic performance and cold endurance throughout migration (Le Pogam et al., 2021). Overall, although all traits did not show the same pattern, our results suggest that snow buntings indeed maintain winter-like cold endurance during migration and during the pre-breeding period in the Arctic.

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[1] http://climat.meteo.gc.ca

TABLE 3 | Linear mixed-effects models comparing phenotypic traits in snow buntings measured during wintering at Rimouski and during pre-breeding at Alert.

Variable	**Body mass**			**Fat score**		
	df	***F***	***P***	**df**	***F***	***P***
Period	1, 626	3.53	0.06	1, 735	1.08	0.30
Sex	1, 793	27.19	<0.0001	1, 802	0.0054	0.94
Sex × Period				1, 802	3.45	0.06
Structural size	1, 792	60.57	<0.0001			
Time at capture	1, 790	8.79	0.003	1, 800	4.83	0.03

Variable	**Hematocrit**			**Pectoralis muscle thickness**		
	df	***F***	***P***	**df**	***F***	***P***
Period	1, 26	11.96	0.002	1, 9	5.24	0.05
Keel				1, 98	380.54	<0.0001

Variable	**M_{sum}**			**BMR**		
	df	***F***	***P***	**df**	***F***	***P***
Period	1, 20	1.32	0.26	1, 92	87.23	<0.0001
Mass	1, 63	13.71	0.0005	1, 108	55.50	<0.0001
Length of captivity				1, 129	6.90	0.01

Models also considered the effect of sex on body mass and fat scores and included covariates meaningful to specific dependent variables. See text for details.

Thermogenic Capacity, Body Mass, and Fat Stores Remain High Up to the Breeding Grounds

We found that body mass, fat scores, and summit metabolic rate were globally comparable between wintering and pre-breeding stages in snow buntings. These results are consistent with the hypothesis that buntings likely maintain winter-level cold endurance and energy stores throughout migration and certainly into early spring on the breeding grounds as suggested previously by Le Pogam et al. (2021). In fact, pre-breeding birds at Alert faced environmental conditions colder than those encountered during winter at Rimouski (**Table 2**). Therefore, the maintenance of these traits likely allows buntings to cope with cold and unpredictable conditions during migration and on arrival on the breeding grounds (Ramenofsky and Wingfield, 2006). Winter-level thermogenic capacity could be particularly beneficial for early arriving males that are known to secure the best breeding territories well before the onset of breeding (Meltofte, 1983; Macdonald et al., 2012; Snell et al., 2018).

We also observed that for a given structural size, males tended to be heavier in winter, but did not differ in fat stores between winter and pre-breeding. In contrast, females did not differ in M_b, but did tend to have larger fat reserves during pre-breeding. This observation is similar to that reported by Laplante et al. (2019) in wintering buntings, and suggests that males and females have a different relative body composition in terms of lean and fat mass, possibly due to differing wintering strategies (Macdonald et al., 2016). As male buntings typically arrive before females on the breeding grounds (Cramp and Perrins, 1994; McKinnon et al., 2016; Snell et al., 2018; Montgomerie and Lyon, 2020), they likely face colder temperatures (**Table 2**), which may require adjustments in body composition to sustain thermoregulatory demands that differ from females. For example, life in the cold leads to increased daily food consumption and consequently larger digestive organs (Barceló et al., 2017), which could increase lean body mass with no effect on relative fat storage.

Lower Oxygen Carrying Capacity and Smaller Pectoralis Muscles on the Breeding Grounds

Not all traits representative of a snow bunting winter phenotype (**Table 1**, Le Pogam et al., 2020) were fully similar when contrasting winter and pre-breeding birds. In fact, both pectoralis muscle thickness and oxygen carrying capacity (hematocrit) were lower in pre-breeding individuals than in birds measured during winter. The difference in muscle thickness could potentially be a direct consequence of tissue degradation during migration as observed in several species (Battley et al., 2000; Bauchinger and Biebach, 2001; Bauchinger et al., 2005; Bauchinger and McWilliams, 2010). However, pectoralis muscles in shorebirds have also been reported to decline in size and mass in the first week after arrival at Alert (Morrison, 2006; Vézina et al., 2012) which has been suggested to act as protein stores to facilitate migratory recovery and transition into the breeding phenotype (Morrison, 2006; Vézina et al., 2012). Although the bulk of snow buntings seem to arrive at Alert around the end of May (AL, FV, pers. obs.), which matches our measurements of pre-breeding birds, local sightings have also confirmed that some individuals arrive much earlier (end of April), albeit in very small numbers (AL, FV, pers. obs.). We therefore cannot completely rule out the possibility that some proportion of the pectoralis muscles might be lost post arrival. Interestingly, the smaller pectoralis size of pre-breeding birds was not reflected in lower shivering heat production measured as M_{sum}. However, this is not necessarily surprising since recent studies have suggested that M_{sum} can be upregulated without changes in muscle size (Stager et al., 2015; Barceló et al., 2017; Milbergue et al., 2018), including in snow buntings (Le Pogam et al., 2020).

The reduced oxygen carrying capacity (lower hematocrit level) measured in pre-breeding buntings relative to wintering individuals is surprising. Birds faced with high energy demands, such as prolonged exercise for migration (Viscor et al., 1985; Bairlein and Totzke, 1992; Morton, 1994; Piersma et al., 1996) or high thermoregulatory requirements, typically show high oxygen transport capacity (Swanson, 1990b; Morton, 1994; O'Connor, 1996; Le Pogam et al., 2020), and birds measured at Alert were living in a considerably colder environment than those measured in winter. The reason for this discrepancy is not immediately clear. However, other studies have also observed a decline in hematocrit during migration (e.g., Piersma et al., 1996; Landys-Ciannelli et al., 2002; Jenni et al., 2006). One possible explanation suggested by Jenni et al. (2006) is that birds could increase their plasma volume by hemodilution to reduce blood viscosity, which could reduce the heart's energy expenditure during migration.

Physiological Maintenance Costs Are Higher on the Breeding Grounds

We observed that physiological maintenance costs measured as BMR were higher during pre-breeding than during winter. This result contrasts with our predictions (i.e., same or lower BMR at Alert), but matches observations by Le Pogam et al. (2021) who reported a spring related increase in BMR in outdoor captive buntings kept on their wintering range. This elevated BMR was in fact maintained in captive birds throughout the periods corresponding to spring migration and most of breeding (Le Pogam et al., 2021). Several studies on migratory shorebirds have also reported an elevated BMR during the arrival period or breeding season in the Arctic (Kvist and Lindström, 2001; Lindström and Klaassen, 2003; Vézina et al., 2012) and this increase in BMR has been interpreted as a result of high thermoregulatory demands on the breeding grounds (Kersten and Piersma, 1987; Kvist and Lindström, 2001; Jetz et al., 2008). While the species in these studies typically winter in relatively warm environments (Kvist and Lindström, 2001; Lindström and Klaassen, 2003; Vézina et al., 2012), the snow buntings compared here were also exposed to colder temperatures in the spring at Alert than in eastern Québec in winter. As far as we know, this is the first report of high maintenance costs at Arctic latitudes in a pre-breeding passerine species. Furthermore, as BMR increased independently from body mass, our results suggest that the higher maintenance costs in the Arctic results from tissue level metabolic activity (i.e., metabolic intensity, Swanson et al., 2017). Interestingly, as this result was also observed in snow buntings unable to migrate and exposed to much warmer temperatures, it could be that some of the underlying variation is driven by endogenous circannual cycles (Vézina et al., 2011; Karagicheva et al., 2016).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the Université du Québec à Rimouski (Rimouski: CPA-54-13-130 and CPA-71-17-195, Alert: CPA-61-15-163 and CPA-71-17-194).

AUTHOR CONTRIBUTIONS

AL and FV conceived the ideas and designed the methodology. AL, M-PL, JD, LR, GR, and FV collected the data. AL analyzed the data. AL, RO'C, FV, and OL led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.724876/full#supplementary-material

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REVIEW
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How Birds During Migration Maintain (Oxidative) Balance

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Animals dynamically adjust their physiology and behavior to survive in changing environments, and seasonal migration is one life stage that demonstrates these dynamic adjustments. As birds migrate between breeding and wintering areas, they incur physiological demands that challenge their antioxidant system. Migrating birds presumably respond to these oxidative challenges by up-regulating protective endogenous systems or accumulating dietary antioxidants at stopover sites, although our understanding of the pre-migration preparations and mid-migration responses of birds to such oxidative challenges is as yet incomplete. Here we review evidence from field and captive-bird studies that address the following questions: (1) Do migratory birds build antioxidant capacity as they build fat stores in preparation for long flights? (2) Is oxidative damage an inevitable consequence of oxidative challenges such as flight, and, if so, how is the extent of damage affected by factors such as the response of the antioxidant system, the level of energetic challenge, and the availability of dietary antioxidants? (3) Do migratory birds 'recover' from the oxidative damage accrued during long-duration flights, and, if so, does the pace of this rebalancing of oxidative status depend on the quality of the stopover site? The answer to all these questions is a qualified 'yes' although ecological factors (e.g., diet and habitat quality, geographic barriers to migration, and weather) affect how the antioxidant system responds. Furthermore, the pace of this dynamic physiological response remains an open question, despite its potential importance for shaping outcomes on timescales ranging from single flights to migratory journeys. In sum, the antioxidant system of birds during migration is impressively dynamic and responsive to environmental conditions, and thus provides ample opportunities to study how the physiology of migratory birds responds to a changing and challenging world.

Keywords: oxidative balance, bird migration, antioxidants, ecophysiological plasticity, exercise physiology

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WHY UNDERSTANDING THE ANTIOXIDANT SYSTEM MATTERS FOR BIRDS DURING MIGRATION

Mitochondria generate the cellular energy required by birds (and all animals) to fuel their basal metabolism and all other activity, including, for example, the impressive long-duration flights of migrating birds. As the cellular powerhouse, mitochondria are also the major site for generation of reactive species (RS), important molecules for signaling across the immune, inflammatory and antioxidant pathways (Dröge, 2002; Ristow et al., 2009; Sindler et al., 2013; McWilliams et al., 2020). However, at higher levels, RS can be deleterious by interacting with and stealing electrons from surrounding molecules (Halliwell and Gutteridge, 2007; Costantini, 2014). Thus, an effective antioxidant protection system has evolved to reduce or avoid the negative physiological effects of RS production, and the components of this system are shared by all eukaryotes (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). Most pertinent to our review of how birds during migration maintain oxidative balance are several key aspects of animal physiology. First, increased metabolism, like that required for long-duration flapping flight, is associated with increased RS production that may overwhelm the antioxidant system, disrupt signaling, and cause oxidative imbalance or 'stress' and thus damage to lipids, proteins, and DNA in cells (Costantini, 2014; McWilliams et al., 2021). Second, the initiation and maintenance of an endogenous antioxidant response is likely energetically expensive (Costantini, 2014); thus, understanding how the antioxidant system of migrating birds contends with RS production is related to the energy costs of migration. Third, physiological demands change with the seasons, and thus understanding the pace and extent of these responses (i.e., phenotypic flexibility) is key to determining how birds contend with the challenges of migration in dynamic seasonal environments.

Birds have often been portrayed as exceptional vertebrates, given that they display relatively high metabolic rates and an associated increase in RS production, yet they are remarkably long-lived compared to mammals (Barja, 1998; Buttemer et al., 2010; Munshi-South and Wilkinson, 2010; Jimenez et al., 2019). Birds are also exceptional in that they primarily fuel high-intensity endurance exercise such as migratory flights by metabolizing fat (McWilliams et al., 2004; Jenni-Eiermann, 2017; Guglielmo, 2018) which has potential acute oxidative costs (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). Fats, and especially polyunsaturated fats (PUFAs) that are common in birds during migration (Pierce and McWilliams, 2005, 2014; Guglielmo, 2018), are highly susceptible to oxidative damage (Hulbert et al., 2007; Montgomery et al., 2012; Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). The bird taxa that comprise 95% of extant species and which have the highest metabolic rates (Neoaves) all share a unique mutation in the major antioxidant regulation pathway that lowers the risk of macromolecular oxidative damage (Castiglione et al., 2020). Specifically, an ancestral mutation in the protein KEAP1, which suppresses the action of the transcription factor NRF2 (NF-E2-related factor 2), permits NRF2 to remain at elevated levels in the nucleus and activate expression of a host of antioxidant target genes (Castiglione et al., 2020). *In vivo* and *in vitro* experiments (Castiglione et al., 2020) demonstrate that this enhanced response lowers the risk of macromolecular oxidative damage and thus likely decreases oxidative stress. This higher baseline activation of the antioxidant response is potentially costly, although consumption of dietary antioxidants could reduce or alleviate the costs of upregulating the antioxidant response even further to contend with an oxidative challenge like migration.

In addition to the adapted master antioxidant response, birds rely on a multifaceted set of context-dependent regulatory mechanisms to quickly respond to the oxidative challenges associated with high-intensity flight fueled by oxidatively vulnerable fats. For example, they can upregulate endogenous antioxidant enzymes as well as synthesize endogenous sacrificial molecules (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). They are also able to obtain exogenous antioxidants from their diet, which is especially pertinent for birds at stopover sites in the fall (Alan et al., 2013; Bolser et al., 2013). These various components of the antioxidant system (**Figure 1**) each have their own function and may interact in concert or independently depending on their location in the body, the type of RS generated, available antioxidant precursors or substrates, and exogenous resources.

One of the challenges of studying how the antioxidant system of birds responds to the physiological challenges of migration is that the primary instigator of the oxidative challenge, increased RS production with increased metabolism, is not yet measurable in whole organisms (Cooper-Mullin and McWilliams, 2016; Costantini, 2016, 2019). Rather, oxidative status must be inferred from measurements of key components of the antioxidant system (e.g., upregulation of antioxidant enzymes implies a response to increased RS production) and ideally simultaneous measures of RS-associated damage (e.g., an increase in lipid peroxidation products implies increased RS production that is insufficiently quenched by the antioxidant system). The primary ways in which RS are generated and how key components of the endogenous antioxidant system provide protection are shown in **Figure 2**. Another challenge of studying phenotypic flexibility in the antioxidant system of migratory birds is that the major site for generation of RS is the mitochondria, which will vary in density and activity across tissues (Costantini, 2019), as well as with age and exercise (Cooper-Mullin and McWilliams, 2016; Jimenez, 2018; Stier et al., 2019; Cooper-Mullin et al., 2021). In addition, RS can act as both damaging molecules and also as signaling molecules that can stimulate the endogenous antioxidant system and immune system (Halliwell and Gutteridge, 2007; Costantini, 2014, 2019; Cooper-Mullin and McWilliams, 2016). There are also a multitude of tests available to probe the response of the antioxidant system to ecologically relevant challenges, too often complicating comparisons across studies (Costantini, 2011, 2016, 2019; Skrip and McWilliams, 2016). Consequently, integrative studies (from mitochondria to cells to tissues to whole organism) that are also comparative (e.g., multiple tissues within the same individuals, migration-state vs. non-migration periods, multiple species that differ in migration strategy) are required to fully understand the antioxidant system of migratory birds within an ecological context. Given that such integrative and comparative studies of migratory birds are largely lacking, evidence to date provides a more piecemeal view of how the antioxidant system of migratory birds changes during migration.

In this review, we focus on the extent to which birds during migration modulate their antioxidant capacity to protect against overwhelming oxidative damage caused by storing and burning fats during long-duration, energy-demanding flights (Skrip et al., 2015; Cooper-Mullin and McWilliams, 2016;

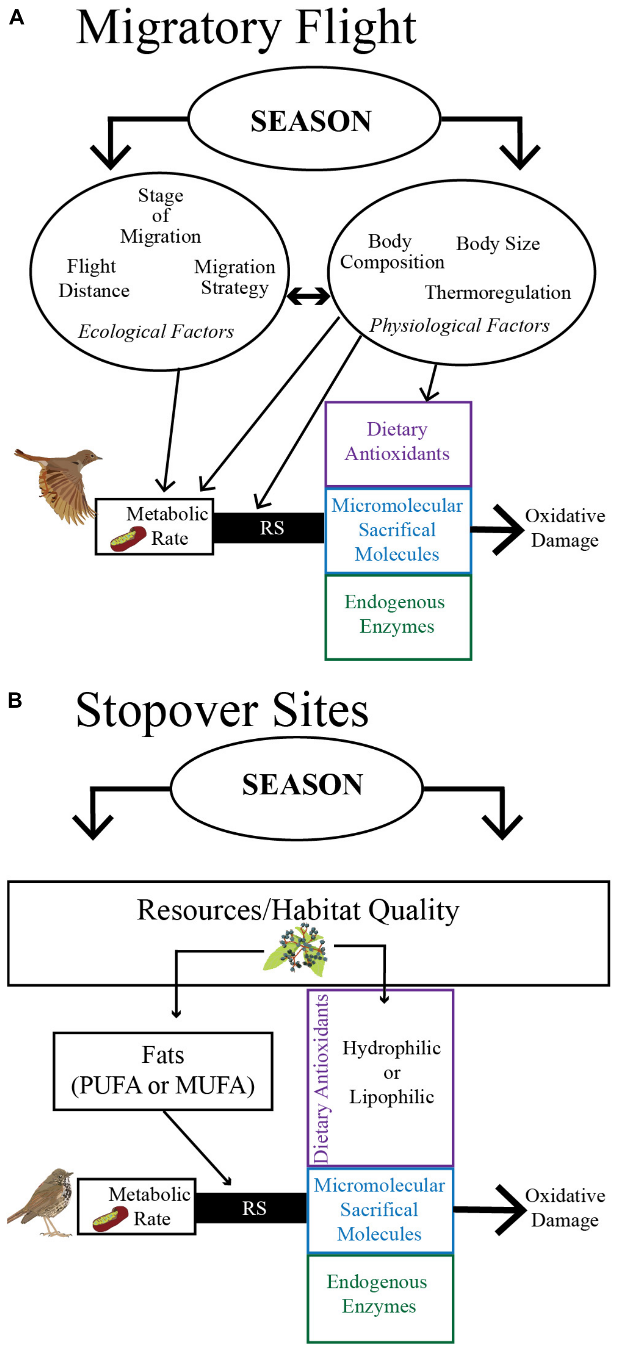

FIGURE 1 | During migration, a multitude of ecological and physiological factors that may be different between seasons (fall vs. spring migration) can alter a bird's metabolic rate and subsequent reactive species (RS) production, as well as how the multifaceted antioxidant system responds to RS production during endurance flight **(A)** and during recovery from flight and preparations for the next flight while at a stopover site **(B)**. The multifaceted antioxidant system consists of ingested dietary antioxidants, micromolecular sacrificial molecules (e.g., uric acid, a byproduct of protein catabolism), and endogenous enzymes that may respond to the production of RS produced primarily in mitochondria. Here, we layout some examples that likely underly observed variation in measurements of oxidative status during migratory flight and stopover. **(A)** The increase in metabolic rate during flight is influenced by *(Continued)*

FIGURE 1 | (Continued)
ecological factors (e.g., distance between stopovers and barriers encountered during flight) and physiological factors (e.g., fat and protein and antioxidant stores, metabolic requirements including thermoregulation) that can affect RS production as well as antioxidant capacity and thus observed oxidative damage. **(B)** Habitat quality at a stopover site governs the resources available for refueling. For example, the amount and type of long-chain polyunsaturated fats (PUFA) or monounsaturated fats (MUFA) a bird consumes and stores during stopover can directly influence an individual's RS production. Additionally, the amount and types of dietary antioxidants available (e.g., fruit abundance in the fall) influences an individual's ability to rebuild antioxidant capacity at a stopover site. Thus, resources available at stopover sites (e.g., dietary antioxidants, fats, and proteins), as well as physiological factors (not shown), influence the rate and extent of recovery from the previous flight and preparations for the next flight.

Skrip and McWilliams, 2016). Other reviews have focused on the regulation of oxidative balance as an underlying driver of aging and longevity (Montgomery et al., 2012; Selman et al., 2012), as determining the sublethal effects of environmental contaminants (Bursian et al., 2017a; Dorr et al., 2019) including ingestion of industrial oil from oil spills (Bursian et al., 2017b; Pritsos et al., 2017), and as an important general indicator of bird health for field ornithologists (Cooper-Mullin and McWilliams, 2016; Hutton and McGraw, 2016; Skrip and McWilliams, 2016). Here, we review in turn the evidence from field and captive bird studies that address the following questions: (1) Do migratory birds build antioxidant capacity as they build fat stores in preparation for long flights? (2) Is oxidative damage an inevitable consequence of oxidative challenges such as flight, and, if so, how is the extent of damage affected by factors such as the response of the antioxidant system, the level of energetic challenge, and the availability of dietary antioxidants? (3) Do migratory birds 'recover' from the oxidative damage accrued during long-duration flights, and, if so, does the pace of this rebalancing of oxidative status depend on the quality of the stopover site? After reviewing the evidence to date, we provide some suggestions for future research that include much needed integrative and comparative studies.

DO BIRDS BUILD ANTIOXIDANT CAPACITY AS THEY BUILD FAT STORES IN PREPARATION FOR MIGRATION?

The course of migration for most birds (**Figure 3**) involves an initial period of over-feeding (called hyperphagia) that enables birds to store appreciable energy, mostly as fat (90+% of body mass increase) as well as key nutrients including protein (King and Farner, 1965; Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004; Bishop and Butler, 2015; Guglielmo, 2018). These periods of intensive feeding and fattening are often associated with diet switching, most notably to fruits during fall migration (Rybczynski and Riker, 1981; Parrish, 1997; Smith et al., 2007, 2015; Carlisle et al., 2012). The duration of the intensive feeding and fattening periods can last a few days to several weeks (Schaub and Jenni, 2001; Chernetsov, 2006; O'Neal et al., 2018), and is influenced by a variety of ultimate and proximate

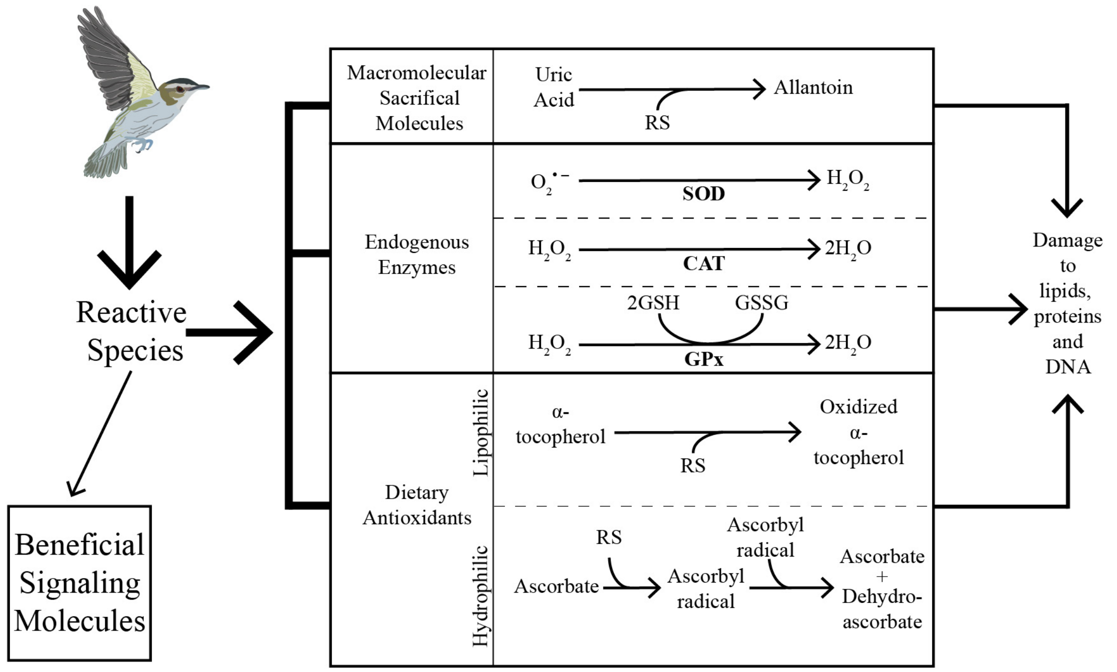

FIGURE 2 | Reactive species (RS) produced during metabolism can serve as beneficial signaling molecules although when in excess RS can cause damage to a variety of important molecules. The multifaceted antioxidant filter of birds can mitigate damage in several ways. The antioxidant filter consists of micromolecular sacrificial molecules, endogenous enzymes and dietary antioxidants. Uric acid is an example of a sacrificial molecule that donates an electron to RS and is oxidized to allantoin. The ratio of uric acid to allantoin can be measured and used as an indicator of oxidative balance. Glutathione peroxidase (GPx) is an example of an endogenously produced enzyme that catalyzes the glutathione (GSH) to glutathione disulfide (GSSG) cycle. During the reaction, GSH donates an electron to RS, and is oxidized to GSSG, which can be recycled back to GSH by glutathione reductase. Superoxide dismutases (SODs) and catalase (CAT) are two other important groups of endogenous antioxidant enzymes that operate in different cells and tissues to quench RS such as oxygen radicals. Vitamin E (α-tocopherol) is an example of a lipophilic antioxidant from the diet, and vitamin C (ascorbate) is an example of a hydrophilic antioxidant from the diet. As RS production increases during endurance flight, the antioxidant system must also be upregulated to prevent an increase in damage.

factors such as migration strategy (e.g., long-distance and short-distance), weather, food availability, predation risk, endogenous cycles, and physiological state (Jenni and Schaub, 2003; Fusani et al., 2009; Goymann et al., 2010; Guillemette et al., 2012; Hou and Welch, 2016). Furthermore, migratory birds exhibit behavioral and physiological adjustments (e.g., reduced heart rate and body temperature, less active when not feeding) during hyperphagia that help to promote fattening by keeping their daily energy expenditure similar to or below non-hyperphagic periods (Carpenter and Hixon, 1988; Butler and Woakes, 2001; Wojciechowski and Pinshow, 2009; Guillemette et al., 2012). Birds during migration then alternate between fasting while flying and feeding and refueling upon arrival at migratory stopover sites in preparation for subsequent migratory flight(s). These rapid changes in feeding rate and food quality over short periods of time (i.e., hours to days) affect the structure and function of the digestive system in migratory birds (McWilliams et al., 2004; McWilliams and Karasov, 2014; Griego et al., 2021) and thus the dynamics of fat (and protein) metabolism and potentially the antioxidant system over the course of many flights and stopovers that constitute a typical migration in birds (**Figure 3**).

Birds are also able to enhance their use of stored fats during migratory flights by changing the 'quality' of their fat stores to enhance the rate of mobilization and oxidation (McWilliams et al., 2021). Fatty acid composition of stored fat changes in birds during migration primarily in response to diet and to a lesser extent preferential metabolism of certain fatty acids (Pierce and McWilliams, 2005, 2014). Such changes in fat quality can affect flight performance during long-duration flights (Price, 2010; Carter et al., 2018; Guglielmo, 2018; McWilliams et al., 2020) as well as levels of oxidative damage (McWilliams et al., 2021). Several experimental and field studies suggest that the quality of fats consumed and stored by migratory birds affects circulating markers of oxidative damage. For example, European Starlings (*Sturnus vulgaris*) fed for many months on diets with more or less $n-6$ PUFA (which then corresponded to the makeup of their fuel stores) exhibited significantly different levels of oxidative damage to lipids (plasma d-ROMs); specifically, birds composed of more n-6 PUFA had consistently higher damage (McWilliams et al., 2020). Similarly, captive White-throated Sparrows (*Zonotrichia albicollis*) that consumed diets with more PUFA had increased circulating oxidative damage (d-ROMs) (Alan and McWilliams, 2013). Common Blackbirds (*Turdus merula*) captured at a stopover site in northern Germany in autumn had a higher plasma fatty acid (FA) peroxidation index and higher non-enzymatic antioxidant

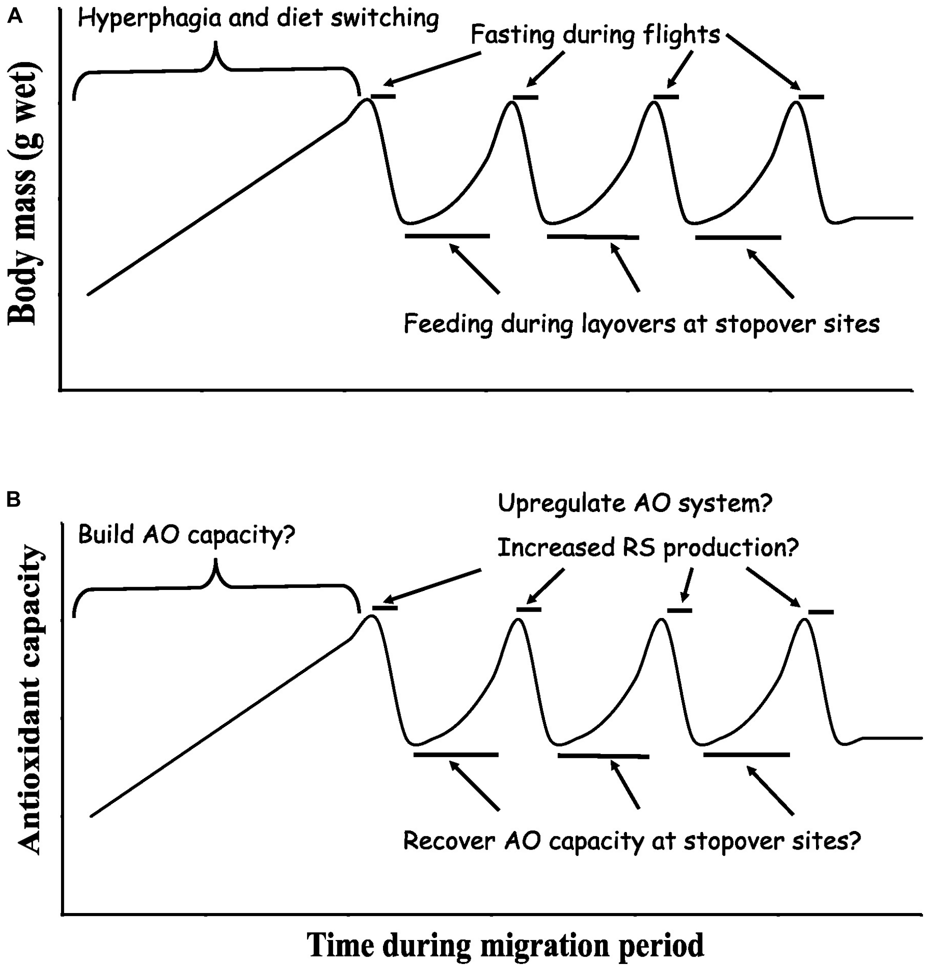

FIGURE 3 | The time course of migration for birds typically involves a period of preparation in anticipation of migration, and then a series of flights while fasting alternating with periods of stopover, refeeding, and refueling along the way. **(A)** In preparation for migration, birds accumulate fat stores primarily by eating more (hyperphagia) and by carefully selecting their diets. Then during a given migration period, a typical migratory bird traveling between wintering and breeding areas must take several flights that are interspersed with layovers at stopover sites where the bird refuels in preparation for the next flight. Thus, birds during migration alternate between periods of high feeding rate at migratory stopover sites and periods without feeding as they travel between stopover sites. **(B)** Possible dynamics of the antioxidant (AO) system over the course of migration. This review outlines the evidence to date for such proposed changes in the antioxidant system of migratory birds.

capacity (Ferric Reducing Antioxidant Power, FRAP, and uric acid) but similar lipid peroxidation (malondialdehyde concentration, or MDA) compared to sympatric resident blackbirds (Eikenaar et al., 2017).

Given that fats, particularly PUFAs, are oxidatively vulnerable, fattening in preparation for migration might be expected to increase RS production and an associated antioxidant response. Evidence to date indicates that migrating birds prepare for the challenge of increased RS and that the response is condition-dependent. For example, amount of stored fat (as measured by either fat score or fat mass) was positively correlated with non-enzymatic antioxidant capacity (plasma OXY) in Blackpoll Warblers (*Setophaga striata*) and Red-eyed Vireos (*Vireo olivaceus*) at a New England stopover site during fall migration (Skrip et al., 2015), and Garden Warblers (*Sylvia borin*) at a Mediterranean coastal stopover site during spring migration (Costantini et al., 2007). However, no such correlation was found in Barn Swallows (*Hirundo rustica*) at the same Mediterranean coastal stopover site during spring migration (Costantini et al., 2007) nor in Northern Wheatear (*Oenanthe oenanthe*) at a stopover site on Helgoland in the North Sea during fall migration (Eikenaar et al., 2020a). Interestingly, stored fat was also positively correlated with oxidative damage (plasma d-ROMs), suggesting that fat stores and/or fattening may incur oxidative costs. Captive bird work further suggests that actively refueling birds prepare for oxidative challenges. Captive-reared Northern Wheatears that were photostimulated into 'migration state' in fall increased their non-enzymatic antioxidant capacity over time as compared to control (*ad libitum*-fed) birds. This increase in non-enzymatic antioxidant capacity was largely due to increases in uric acid, as indicated by highly correlated FRAP and uric acid assays (Eikenaar et al., 2016). However, the high variability in lipid peroxidation (red blood cell MDA) produced no consistent differences between control and migration-state birds (Eikenaar et al., 2016).

The studies we describe above have examined the oxidative status of songbirds that were either sampled during migration or held in captivity and photostimulated into migratory state. The only studies to date that directly compare the oxidative status of birds preparing for migration versus during other stages of their annual cycle (e.g., wintering) have been performed in a shorebird species, the Hudsonian Godwit (*Limosa haemastica*), and a migratory quail, the Common Quail (*Coturnix coturnix*). Gutiérrez et al. (2019) sampled blood from free-living godwits inhabiting an island in southern Chile at three time periods from January to March (wintering, fuelling, and pre-departure) and found that pre-departure birds preparing for their >10,000-km northbound migratory flight increased total antioxidant capacity (TAC) and reduced oxidative damage (TBARS), without changing enzyme activity (cytochrome c oxidase and citrate synthase). Marasco et al. (2021) compared oxidative status of captive quail during the migratory and non-migratory phases, the former phase induced using photoperiod manipulations that simulated autumn migration. In contrast to free-living godwits preparing for spring migration, the migratory-phase quail had higher levels of oxidative damage (TBARS) compared to non-migratory birds (in liver but not red blood cells or pectoral muscle), and no comparable measure of total antioxidant capacity was reported. In addition, whole-tissue enzyme assays revealed lower SOD activity in red blood cells and liver (but not pectoral muscle), and higher GPx activity in pectoral muscle (and sex-specific differences in red blood cells and liver). Marasco et al. (2021) suggested that the higher GPx activity and lower TBARS they found in muscles compared to liver of migratory-phase captive quail may indicate tissue-specific preferential protection, with pectoral muscle being "spared" from damage to support migratory flight. Determining whether the results from these two longitudinal studies are related to species-specific differences (e.g., life history and migration strategy), experimental conditions (e.g., captive vs. free-living birds), tissues sampled and assays used, or migratory season (i.e., autumn vs. vernal) requires many more such studies.

In sum, migratory birds seem to build some component of their antioxidant capacity concomitantly with fat stores (as seen in Blackpoll Warblers, Red-eyed Vireos, Garden Warblers, Barn Swallows, Northern Wheatears, Common Blackbirds, and Hudsonian Godwits). Furthermore, increased oxidative damage is a likely cost of increasing (or maintaining more) fat stores (as seen in Blackpoll Warblers, Red-eyed Vireos, Garden Warblers, Barn Swallows, and in liver of Common Quail), especially if these fats are composed of mostly PUFA (as seen in European Starlings and White-throated Sparrows). However, higher damage may not be inevitable as birds fatten prior to migration (as seen in Northern Wheatears, Common Blackbirds, Hudsonian Godwits), perhaps because different tissues, individual birds or bird species vary in the extent to which they build antioxidant capacity.

IS OXIDATIVE DAMAGE AN INEVITABLE CONSEQUENCE OF OXIDATIVE CHALLENGES DURING A FLIGHT?

The metabolic costs of flying are long established (Bishop and Butler, 2015) as are the many physiological adjustments that enable the long-duration flights achieved by birds during migration, including increased oxidative enzymes (McClelland, 2004; Weber, 2011; Banerjee and Chaturvedi, 2016; Dick and Guglielmo, 2019; Carter et al., 2021) and fatty acid transport and oxidation (Guglielmo et al., 2002; McWilliams et al., 2004, 2021; Guglielmo, 2018), as well as hypertrophy of key organs such as pectoralis and liver (Marsh, 1984; Dietz et al., 1999; Piersma et al., 1999; Lindström et al., 2000; DeMoranville et al., 2019). These biochemical adjustments are associated with upregulation of genes responsible for regulating metabolism (PPARγ and PPARα), and key genes responsible for fat transport (FABPpm, CD36, and H-FABP) and fat oxidation (ATGL, LPL, and MCAD) among others (McFarlan et al., 2009; Zhang et al., 2015; Corder et al., 2016; DeMoranville et al., 2019). Furthermore, experimental studies demonstrate that long-distance flight training in a wind-tunnel upregulates genes involved in mitochondrial metabolism and fat utilization in pectoralis but not liver (DeMoranville et al., 2020). Below we review whether such metabolic upregulation is associated with oxidative challenges during a given flight and whether flight specifically results in oxidative damage and reduced antioxidant capacity measured immediately after.

Does Flight Elevate Oxidative Damage?

Despite the importance of oxidative damage measures to understanding tradeoffs during migration, relatively few studies have measured the acute effects of flight on byproducts of RS production. Moreover, these studies have been spread across different study systems and measures of oxidative damage. As outlined below, the effects of acute flight on oxidative damage are more consistent in experimental studies, where flight time and speed are controlled, compared to field studies of free-living birds, and the extent of reported damage seems related to the extent of antioxidant system response. A commonly used measure of oxidative damage has been the d-ROMs test (Costantini, 2016; Skrip and McWilliams, 2016), which is most closely associated with lipid peroxidation but can reflect hydroperoxides of any origin (Davies, 2016; Ito et al., 2017). This assay is responsive to changes in oxidative status over a relatively short time scale (e.g., minutes to hours) and uses blood serum, making it both non-destructive and a more general indicator of circulating lipid peroxidation products than other more tissue-specific measures (e.g., in muscle; Abuja and Albertini, 2001; Colombini et al., 2016) and compared to other specific assays (e.g., MDA, see below).

Oxidative damage to lipids has usually been found to not change or has increased after flight across the ca. dozen studies to date, and this variation in response can be explained in part by the extent of response of the antioxidant system to the physiological challenges of the flight. In one of the first studies to investigate the acute effects of flight, pigeons (*Columbia livia*) flown for long durations (310 ± 31 min) had 54% higher lipid damage than in birds flown short durations (80 ± 15 min) or not flown (Costantini et al., 2008). In contrast, flight duration (range: 65–205 min) was not related to d-ROMs levels in Northern Bald Ibis on a managed migration (*Geronticus eremita*; Bairlein et al., 2015). Similarly, several wind-tunnel studies on starlings (*S. vulgaris*) that involved weeks of flight training leading to long final flights up to 6 h have not found any change in d-ROMs associated with flight, either within individuals pre- and post-flight or between individuals that were either flown or not flown (McWilliams et al., 2020; Frawley et al., 2021). In contrast, starlings involved in a similar 2-week wind-tunnel flight training decreased d-ROMs after flight compared to pre-flight indicating a potential hormetic response (DeMoranville, 2020). Zebra Finches (*Taeniopygia guttata*) that underwent 3 h of short-burst flight, a flight type that is more aerobically intense than sustained flight (Nudds and Bryant, 2001), showed a significantly greater increase in d-ROMs over those 3 h than unflown birds (Costantini et al., 2013). Furthermore, birds flown at the greatest speed intensity (0.9 km/h) had the highest levels of d-ROMs and birds flown at a slower speed (0.3 km/h) had intermediate levels (Costantini et al., 2013). Over a longer period of flight training, Zebra Finches flown for 2 h at 2.4 km/h per day consistently showed no acute changes in oxidative damage to lipids on a given day of flight training, and no difference in d-ROMs was detected between trained and untrained individuals over 1.5 months of daily flying (Cooper-Mullin et al., 2019). However, these same individuals exhibited a consistent increase in an antioxidant enzyme (GPx) that may have protected them against oxidative damage. Finally, correlational field studies of free-living, migrating Garden Warblers (*S. borin*; Skrip et al., 2015) and Nathusius' bats (*Pipistrellus nasthusii*; Costantini et al., 2018) found that d-ROMs were highest in plasma samples taken most recently after migratory flight, while a study that manipulated the effort of flight by plucking wing feathers of Great Tits (*Parus major*; Vaugoyeau et al., 2015) found no difference in d-ROMs between handicapped and control individuals. Some of this inconsistency in results between studies seems related to the extent of flight costs and duration. Notably, the level of oxidative damage measured by the d-ROMs test in both European Starlings and Yellow-rumped Warblers (*Setophaga coronata*) flown for known durations in wind-tunnel experiments was highest in individuals flown for longer and that had higher energy expenditure during flight (Dick and Guglielmo, 2019; DeMoranville, 2020). Likewise, free-living Red-eyed Vireos had lower oxidative damage compared to Blackpoll Warblers caught on an island stopover site (Block Island and Rhode Island), presumably because the warblers had to fly farther to reach the stopover site (Skrip et al., 2015).

Given the susceptibility of PUFA to oxidative damage (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016), some studies use the concentration of malondialdehyde (MDA) to assess oxidative damage because it is a relatively stable peroxidation byproduct of long-chain PUFAs that are common in cell membranes (Halliwell and Gutteridge, 2007; Ayala et al., 2014). MDA levels were elevated in blood samples taken from Budgerigars (*Melopsittacus undulatus*) 24 h after completing 5 days of mild escape-flight training, although this effect was largely dependent on whether or not individuals had been supplemented with antioxidants (Larcombe et al., 2008). Conversely, natural variation in aerobic activity (including flight) of Budgerigars was unrelated to MDA concentrations (Larcombe et al., 2015). Likewise, MDA concentration did not differ between Common Blackbirds presumed to be year-round residents versus presumed to have recently completed a migratory flight (Eikenaar et al., 2017). Therefore, studies that examine oxidative damage to lipids (i.e., d-ROMs and MDA) show varied responses to flight, likely due to variation in flight intensity and the extent to which a bird's antioxidant system responds in preparation for and during flight.

Oxidative damage to protein, measured as the concentration of protein carbonyls (Halliwell and Gutteridge, 2007; Davies, 2016), has also been assessed in response to flight given the potential for muscle damage (Guglielmo et al., 2001; Dick and Guglielmo, 2019). Plasma protein carbonyls were significantly higher in Zebra Finches flown for 3 h at 0.9 km/hr compared to individuals flown at 0.3 km/hr or unflown individuals (Costantini et al., 2013). Similarly, concentration of protein carbonyls in the flight muscle of Yellow-rumped Warblers was positively related to the duration of flight in a wind tunnel and was significantly higher in individuals flown for 6 h than for unflown individuals (Dick and Guglielmo, 2019). Meanwhile, in a field study on European Robins (*Erithacus rubecula*) during migration, plasma concentrations of protein carbonyls were highest among birds caught at night and therefore presumably directly out of endurance flight (Jenni-Eiermann et al., 2014).

In sum, studies on the acute effects of flight on oxidative damage provide evidence for an increase in damage after flight (as assessed using d-ROMs, MDA, and protein carbonyls) but certainly not in all cases. Only protein carbonyls have consistently increased in response to flight, although there are few studies to date that have used this measure. Clearly, levels of oxidative damage are dynamic and seem responsive to a host of ecologically relevant factors, including duration of flight and diet, which complicates comparisons between studies especially when different assays are used.

Does Flight Deplete Antioxidant Capacity?

Birds can protect themselves from exercise-induced increases in RS production using a robust endogenous antioxidant system (**Figure 1**). Whether a bird depletes its antioxidant capacity, or increases it in response to flight, may depend on ecologically relevant factors such as individual condition (e.g., fat stores and oxidative damage levels), access to dietary antioxidants, and the amount of exercise. Enzymatic and non-enzymatic antioxidants may also respond differently to acute oxidative challenges, indicating that these components may serve different or complimentary roles in the antioxidant system.

Enzymatic antioxidants, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), are the most commonly assayed enzymes in avian studies and have been shown in most cases to rapidly increase in activity in response to flight. For example, European Robins caught during nocturnal migratory flight had higher GPx activity compared to birds on stopover (Jenni-Eiermann et al., 2014), and Yellow-rumped Warblers flown in a wind tunnel had higher SOD after flight than individuals at rest (Dick and Guglielmo, 2019). The response of enzymatic antioxidants in flight-trained European Starlings to an approximately 3-h flight in a wind tunnel depended on availability of dietary antioxidants (Frawley et al., 2021). Specifically, starlings fed a diet not supplemented with anthocyanin, a dietary antioxidant, had decreased GPx activity immediately after flight, whereas GPx activity did not change after flight for starlings fed a diet supplemented with anthocyanins (Frawley et al., 2021). Furthermore, Zebra Finches flown for short bursts had similar serum GPx, CAT, and SOD activity after flight compared to unflown control birds (Costantini et al., 2013). Zebra Finches flown for 2 h had elevated GPx activity immediately after flight, and this acute increase in GPx activity was consistently observed even after ca. 2 and 6 weeks of daily flight, indicating that birds were able to rapidly downregulate GPx activity when the oxidative challenge had passed (Cooper-Mullin et al., 2019). Interestingly, daily flight by Zebra Finches over 1.5 months increased coordination between the enzymatic (GPx) and non-enzymatic components of the antioxidant system (Cooper-Mullin et al., 2019). Furthermore, the inclusion of dietary precursors for these enzymatic antioxidants (e.g., selenium for GPx) can increase the activity of the enzyme during flight (Zigo et al., 2017). The common theme that emerges from these studies is that the activity of enzymatic antioxidants rapidly changes during bird flight, but the extent of these changes is related to the availability of dietary antioxidants and the response of other components of the antioxidant system.

Non-enzymatic endogenous antioxidants include sacrificial molecules such as glutathione (GSH) and uric acid, which arise from a diverse set of metabolic pathways and cycles and have varying metabolic uses (Tsahar et al., 2006; Carro et al., 2012; Cooper-Mullin and McWilliams, 2016). Uric acid, a byproduct of protein catabolism and a powerful antioxidant, has been found to consistently increase after flight in several field and lab studies. European Starlings flown in wind tunnels had elevated serum uric acid immediately after up to 6 h of flight (Carter et al., 2020; McWilliams et al., 2020; Frawley et al., 2021). White-crowned Sparrows (*Zonotrichia leucophrys*) that were flown at high speeds in a hop/hover wheel (Tsahar et al., 2006) and pigeons flown for 4 h (Gannes et al., 2001) also had increased uric acid concentrations after flight. Serum uric acid was also higher in Garden Warblers, Pied Flycatchers (*Ficedula hypoleuca*), and European Robins on active migration compared to birds at rest or refueling on stopover (Jenni-Eiermann and Jenni, 1991). Given that it is a byproduct of protein catabolism during exercise, uric acid may serve as a particularly important antioxidant for flying birds.

The complexity of interpreting uric acid measurements as a marker of non-enzymatic antioxidant capacity can be avoided by using the OXY-adsorbent test, a common serum-based assay that provides a general snapshot of a bird's ability to quench RS. Generally, OXY has been shown to decrease after flight, indicating that birds deplete antioxidant capacity in response to an acute challenge. Pigeons showed a 19% drop in serum antioxidant capacity after being flown for 200 km (ca. 310 ± 31 min, Costantini et al., 2008), and Zebra Finches depleted antioxidant capacity immediately after 2 h of flight and then increased OXY after getting a reprieve from regular daily flying (Cooper-Mullin et al., 2019). European Starlings also reduced OXY (and oxidative damage, noted above) immediately after flights in a wind tunnel, and the extent of this change was more apparent in birds that expended more energy during their longest flights (DeMoranville, 2020). OXY also changed in European Starlings immediately after flights, although the direction and extent of the change depended on season (fall vs. spring migration) and whether diets were supplemented with antioxidants (Frawley et al., 2021).

In summary, there is some evidence that enzymatic antioxidants, particularly GPx, may serve as a first line of defense for birds faced with an oxidative challenge, although research is scarce on the acute responses of other major antioxidant enzymes, particularly CAT and SOD. Likewise, non-enzymatic endogenous antioxidants such as uric acid consistently increase after flight, suggesting they may be functionally important. In contrast, other components of non-enzymatic antioxidant capacity as measured with the OXY test usually decrease after flight, and the extent of this acute change in OXY after flight depends on the acute response of enzymatic antioxidants such as GPx when regular daily flying is prolonged (Cooper-Mullin et al., 2019). Consumption of dietary antioxidants by European Starlings affects the response of enzymatic antioxidants as well as non-enzymatic antioxidants (DeMoranville et al., 2021; Frawley et al., 2021) and has a multitude of related effects: antioxidant consumption reduces corticosterone production in flight-trained birds, and thus potentially protects against the costs of high glucocorticoids (Casagrande et al., 2020), and enhances the breeding condition of males (Carbeck et al., 2018). The role of corticosterone during preparation for migration, in departure during migration (e.g., from stopover sites), and in support of sustained migratory flights has been recently reviewed by Bauer and Watts (2021). Importantly, Cooper-Mullin et al. (2021) used stable isotope-labeled vitamin E to reveal that consumed dietary antioxidants are absorbed and reach muscle mitochondria of Zebra Finches but only when they are regularly exercised (2 h of regular daily flying). Further studies are needed that simultaneously measure multiple components of the antioxidant system and the contribution of dietary antioxidants to better understand how birds respond to the oxidative demands of flight and migration.

Mechanisms of Oxidative Damage and Antioxidant Capacity

When a given flight is associated with an acute increase in oxidative damage and an acute decrease in antioxidant capacity, then the interpretation is relatively straightforward: higher metabolic rate during exercise increases the escape of radicals from the electron transport chain, RS diffuse from the mitochondria to the cytosol and intercellular spaces, and then they are quenched by certain aspects of the antioxidant system. Here we discuss possible mechanisms that can explain other results (e.g., no oxidative damage or change in certain components of the antioxidant system).

When no acute changes in oxidative damage or antioxidant capacity are observed following flight, one possible interpretation is that flight did not increase the amount of radicals escaping from the electron transport chain. There are several, not mutually exclusive, ways that this could be achieved: production of electrons (in the form of reduced nicotinamide adenine dinucleotide, NADH) by the citric acid cycle could be limited by reduced mass-specific activity of the cycle (Tretter and Adam-Vizi, 2005). Alternately, electrons are less likely to escape and form radicals if they are rapidly combined with protons and oxygen to form water. In addition, uncoupling proteins may prevent the formation of large gradients that might produce a scarcity of protons to combine with electrons (Cooper-Mullin and McWilliams, 2016). Electron escape and RS production might also be regulated by the concentration and activity of cytochrome oxidase, which catalyzes the combination of electrons, protons and oxygen (Bode et al., 2013). Although these factors have not been studied extensively in birds or in relation to aerobic activity, variation in RS production across species and tissues could help explain the range of acute responses to flight in birds documented across studies to date.

Inconsistent responses of oxidative damage and antioxidant capacity to flight may also be the result of complex interactions between RS production and the antioxidant system of birds. As a general model, we might assume that RS are continuously produced in animal tissues at a certain rate and that this rate increases during flight (Halliwell and Gutteridge, 2007). Simultaneously, RS and byproducts of RS damage may be neutralized at a certain rate through the actions of antioxidant enzymes, sacrificial molecules, and dietary compounds (**Figure 1**; Cooper-Mullin and McWilliams, 2016). Combined, this means that RS, and therefore measured concentrations of damaged compounds, will accumulate and increase when the rate of RS production is greater than the rate of neutralization and decrease when the rate of neutralization is greater. If the rate of RS neutralization is static, then damage will accumulate throughout the period of flight and then decrease during the following recovery period. However, it is well established that the antioxidant system can flexibly respond and can increase capacity by upregulating the expression of antioxidant enzymes, upregulating the production of sacrificial molecules, and increasing the transport of stored antioxidants into target tissues (Cooper-Mullin and McWilliams, 2016; Costantini, 2019). Moreover, it is reasonable to expect the rate of RS neutralization to be upregulated proportionally with the accumulation of RS and RS byproducts (Dröge, 2002).

In **Figure 4**, we present a conceptual model that demonstrates how the extent of measurable oxidative damage associated with a given flight may depend on the pace and extent of antioxidant upregulation, and the intensity of the flight. In particular, a simple exponential response of neutralization to RS concentration can limit the accumulation of RS during flight and lead to more rapid reductions following flight. Meanwhile, the addition of a lag in response, due to the time required for signaling and the synthesis of antioxidants (Ji et al., 2006; Sthijns et al., 2016), is enough to create a simple hormetic response where accumulated damage decreases in the middle of the flight [**Figure 4**, High RS – AOX (lag)]. Hormesis can also depend on the intensity of an oxidative challenge (Nikolaidis et al., 2012; Costantini, 2014), meaning that milder activity can, at times, produce a muted antioxidant response and therefore higher levels of accumulated RS byproducts [**Figure 4**, Low RS – AOX (lag)]. All of these relatively simple factors can interact to produce considerable variation in measurement of damage, which will depend on the timing of sample collection, the tissue collected, the intensity of activity, and the regulatory responsiveness of the study species. Furthermore, there is evidence that the antioxidant response may differ between seasons and tissues, and that access to dietary antioxidants may boost or attenuate certain antioxidant responses (Frawley et al., 2021) and this can depend on regular exercise (Cooper-Mullin et al., 2019, 2021). In sum, the antioxidant response is not static, and acquiring a better understanding of the pace and extent of antioxidant up- and down-regulation under ecologically relevant situations is needed.

DO MIGRATORY BIRDS ‘RECOVER’ FROM OXIDATIVE DAMAGE ACCRUED DURING LONG-DURATION FLIGHTS?

Birds arriving at a stopover site are faced with the prospect of taking off again in a few days to reach their next stopover or final destination. In many cases, these birds will have experienced oxidative damage from long-distance flight (see previous section), which may have compromised their structural or stored fats and/or proteins. In addition to contending with the consequences of this damage, birds must also build fuel stores to prepare for their next flight. On stopover, therefore, a bird should recover from the damage it has experienced and prepare (both in terms of accumulating energy and antioxidants) for the next oxidatively challenging activity (i.e., flight to the next destination and/or breeding).

In the context of oxidative status, we define ‘recovery’ by migratory birds as a decrease in oxidative damage to fats and proteins and a potential concomitant increase in chiefly non-enzymatic antioxidant capacity. As we describe below, evidence exists that birds do ‘recover’ on stopover, but their capacity to do so may depend on time, a bird’s condition, and the resources it finds at the stopover site. In songbirds, experimental evidence has shown that birds *are what they eat* (the fatty acids they consume are incorporated into fat stores), birds *use what they are* (birds catabolize their fat stores for flight and thermoregulation), and *what birds are* influences physiological performance (Pierce and McWilliams, 2005, 2014; McWilliams et al., 2020). For instance, the molecular make-up of dietary fatty acids contributes to oxidative damage (Cooper-Mullin and McWilliams, 2016; Skrip and McWilliams, 2016). Starlings fed a PUFA-rich diet experienced higher oxidative damage than those fed a MUFA-rich diet (McWilliams et al., 2020), indicating that the type of fat birds

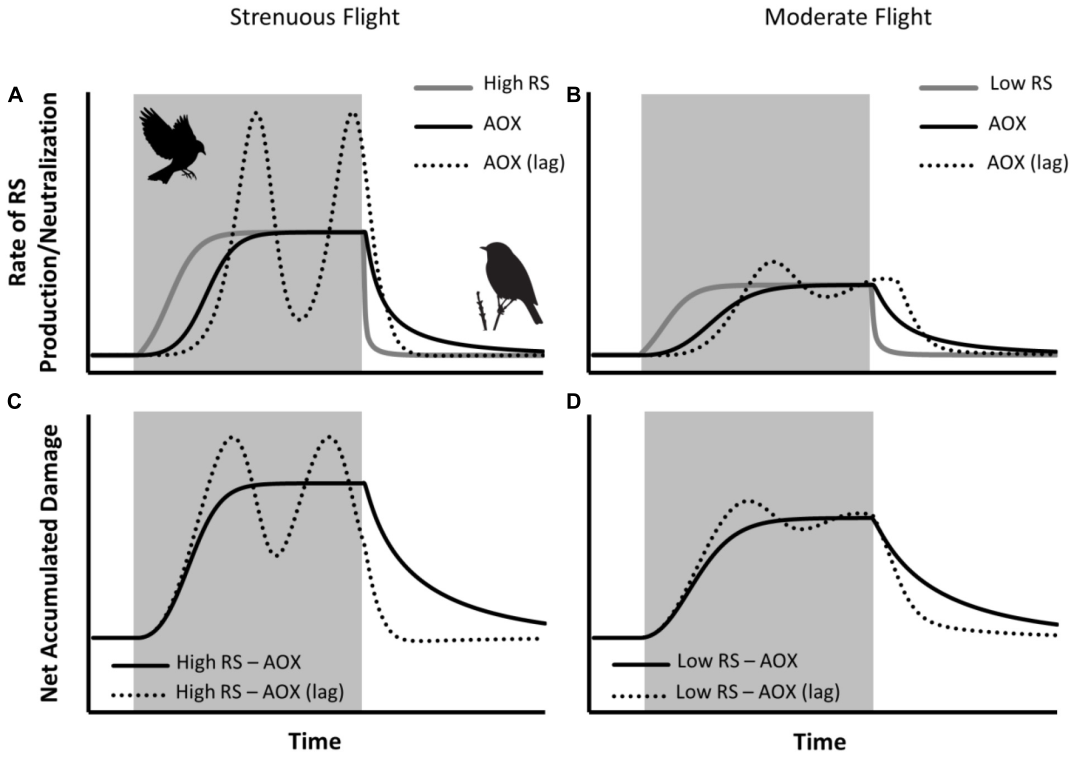

FIGURE 4 | Conceptual model of the accumulation of RS byproducts measured by oxidative damage assays. The cumulative sum of the rates of RS production and neutralization over time **(A,B)** produce the net accumulated oxidative damage at a given time point **(C,D)**. The rate of RS production increases to a set level during flight (shaded areas) and then decreases during recovery, with the set level higher for more strenuous flight **(A)** than moderate flight **(B)**. The rate of scalable antioxidant activity changes exponentially in response to accumulated damage, and therefore rates of RS neutralization (AOX) also vary between strenuous and moderate flight. Moreover, the rate of RS neutralization becomes more variable and increases more dramatically with a greater delay in response time [AOX (lag)]. Subsequent measurements of oxidative damage will depend on the responsiveness of the antioxidant system and the timing of the measurement, with strenuous flight **(C)** sometimes resulting in lower measurements of damage than moderate flight **(D)**.

consume on stopover likely affects the scope of potential recovery from oxidative damage incurred during flight. If birds have access to dietary antioxidants on stopover, then it is likely that these exogenous antioxidants could increase the scope of recovery by, for example, protecting fat stores. However, no study has directly examined this possibility in the wild. Post-flight recovery research on migratory birds is still emerging, characterized by a series of 'firsts.'

Recovery From Oxidative Damage on Stopover

The first evidence of potential acute recovery on stopover came from actively migrating European Robins (*Erithacus rubelica*) caught flying at night through a Swiss mountain pass in autumn (Jenni-Eiermann et al., 2014). Birds captured at night out of flight had higher oxidative damage to proteins (circulating protein carbonyls) than birds captured while resting during the day (Jenni-Eiermann et al., 2014). However, the timeframe over which recovery occurred was unknown, because damage was not tracked over time within individuals. The first study to track oxidative damage in relation to time after a long-distance flight found that circulating oxidative damage to lipids (d-ROMs) decreased among recaptured Garden Warblers the longer they were at a Mediterranean stopover site in spring [between 1 and 192 h (8 days)]; opportunistic intra-individual sampling (*n* = 2) confirmed that damage decreased within birds over a matter of days (Skrip et al., 2015). Taking 'the next logical step' after Skrip et al.'s (2015) cross-sectional finding, Eikenaar et al. (2020b) longitudinally tracked changes in damage within individuals and confirmed that migrating songbirds are capable of recovering from oxidative damage on stopover. Northern Wheatears caught out of spring migration in Germany and held in captivity for 3 days decreased their oxidative damage to polyunsaturated fats (MDA) and appeared to stabilize that damage at a consistent level, suggesting that there may be a threshold of low damage or 'oxidative set-point' (Eikenaar et al., 2020b) that birds seek to reach before taking their next long-distance flight.

Taken together, these three studies provide the first evidence that songbirds are capable of recovering from oxidative damage after flight. How they do so, however, remains an open question. Do birds repair damaged molecules, replace them, or a combination of both? Do resources available at a given stopover site or the physiological condition of an individual bird influence how birds address oxidative damage (**Figure 1**)? The minimum time within which birds begin to recover after a flight is also uncertain. Migrating songbirds held in handling bags for short periods before blood sampling (30 min, Costantini et al., 2007; 20–192 min, Skrip et al., 2015) exhibited no change in oxidative measures. However, Gray Catbirds (*Dumetella carolinensis*) caught during autumn migration and kept overnight had increased uric acid and decreased levels of residual total antioxidant capacity within at least a 12-h period (Cohen et al., 2008a).

Recovery of Antioxidant Capacity on Stopover

Evidence is also mounting that birds are capable of recovering their antioxidant capacity while on stopover. Although time on stopover and circulating non-enzymatic antioxidant capacity (OXY) were not correlated in the cross-sectional analysis of Garden Warblers described above, antioxidant capacity did increase in the two Garden Warblers opportunistically resampled over the course of several days, hinting that such recovery is possible (Skrip et al., 2015). An experimental study with flight-trained European Starlings (DeMoranville, 2020) was the first to demonstrate that songbirds can recover antioxidant capacity after long-distance flight. Starlings depleted their circulating non-enzymatic antioxidant capacity (OXY) during 15 days of nearly daily flight in a wind tunnel (totaling >640 km), but within 2 days of rest (i.e., no flight), birds recovered to their baseline (pre-flight-training) levels independent of whether they were supplemented with water-soluble antioxidants (i.e., anthocyanin) (DeMoranville, 2020). There is evidence that restoration of antioxidant capacity in metabolically active tissues is mediated by the transcription of antioxidant enzyme proteins. For example, during recovery, these same flight-trained starlings had greater mRNA levels of antioxidant enzymes in the liver (CAT, SOD2, and GPX1) and pectoralis (SOD2) compared to unflown starlings, while lipid damage levels were similar (DeMoranville et al., 2021). Like recovery from damage, recovery of antioxidant capacity, however, remains to be mechanistically explained.

Consumption of dietary antioxidants on stopover may contribute to recovery of antioxidant capacity, and migratory birds seem able to select diets that provide fats and antioxidants that can assist or accelerate this recovery (Sapir et al., 2004; Cohen et al., 2009). Migratory songbirds at an offshore New England stopover site during autumn preferred fruits from species with higher fat content and selected fruits rich in dietary antioxidants (Alan et al., 2013; Bolser et al., 2013). Birds can select diets using solely antioxidant content, as shown by a series of food-choice experiments involving Blackcaps (*Sylvia atricapilla*) (Catoni et al., 2008a,b; Schaefer et al., 2014). Given a choice of food supplemented with anthocyanins or not, Blackcaps preferred a supplemented diet (Catoni et al., 2008a; Schaefer et al., 2008). Sources of dietary antioxidants during spring migration are understudied but may include seeds (Cohen et al., 2008b; Beaulieu and Schaefer, 2013), insects (Catoni et al., 2008a; Eeva et al., 2010), leaves (Catoni et al., 2008a), and fruits (Cohen et al., 2009; Alan and McWilliams, 2013; Bolser et al., 2013). It is highly likely that birds take advantage of fruits in tropical areas prior to or during spring migration, as suggested by a geolocator study that found migrating Red-Eyed Vireos make an unexpectedly long stopover in Colombia during spring (Callo et al., 2013).

Recent experiments with actively migrating birds in autumn in New England have revealed the benefits of consuming dietary antioxidants and how antioxidant capacity and fat stores interactively influence the time passerines spend on stopover (Cooper-Mullin and McWilliams, 2021). For example, gavage-feeding dietary anthocyanins to Hermit Thrushes (*Catharus guttatus*) on an *ad libitum* diet boosted their ability to build non-enzymatic antioxidant capacity (OXY; Cooper-Mullin and McWilliams, 2021). Furthermore, birds on an *ad libitum* diet increased non-enzymatic antioxidant capacity (OXY) compared to birds on a diet designed to maintain their weight at capture (Cooper-Mullin and McWilliams, 2021). Studies in mammals have identified anthocyanins as enhancers of the NRF2 antioxidant pathway that activates the expression of antioxidant genes (Shih et al., 2007; Aboonabi and Singh, 2015; Tian et al., 2019; Aboonabi et al., 2020). Dietary antioxidants may also help songbirds increase enzymatic antioxidant capacity by stimulating the same pathway. For example, European Starlings supplemented with anthocyanins had higher mRNA levels of antioxidant enzymes associated with combating reactive species production in the mitochondria (SOD2) and lipid peroxidation in the peroxisome (CAT) in flight muscle compared to unsupplemented birds (DeMoranville et al., 2021). How exogenous and endogenous antioxidants interact in birds on stopover while recovering or refueling still needs more investigation.

The studies described above demonstrate that birds (evidence mostly in songbirds) can decrease oxidative damage and build antioxidant capacity rapidly, in a matter of days after a migratory flight. These studies also demonstrate that dietary anthocyanins (Catoni et al., 2008a; Schaefer et al., 2008), and flavonoids (Catoni et al., 2008b), as well as dietary PUFA (Alan and McWilliams, 2013; DeMoranville et al., 2020; McWilliams et al., 2020), influence a songbird's oxidative status and stopover length (Cooper-Mullin and McWilliams, 2021). However, how the quantity or quality of a bird's fat stores, the energetic demands of previous flights, and the available resources at a stopover aid or hinder this recovery is unclear. The challenge of refueling on stopover is twofold for birds: they have to accumulate fatty acids that make them more efficient fliers (Price, 2010; Ferretti et al., 2019; McWilliams et al., 2020), but they also need to protect those fats as well as recover from the previous flight (Metzger and Bairlein, 2011; Skrip et al., 2015). Future research should focus on the oxidative challenges that recovery and refueling simultaneously pose to migrating birds.

CONCLUSION

The components of the antioxidant system of birds (**Figures 1**, **2**) are broadly similar to that of all aerobic organisms; however, there are several unique aspects of the avian antioxidant system that are important and relevant to our understanding of how birds during migration maintain oxidative balance. First, a unique mutation in the major antioxidant regulation pathway enables

95+% of bird species to maintain a constitutively active master antioxidant response that lowers the risk of macromolecular oxidative damage associated with relatively high metabolic rates (Castiglione et al., 2020). Second, the antioxidant system of migratory birds is able to flexibly and rapidly respond to the oxidative challenges of flying and fasting (**Figure 3**) during the course of migration, with dietary antioxidants and protein catabolism (i.e., uric acid production) playing key roles in maintaining oxidative balance. Given that uric acid is a byproduct of protein catabolism in birds, it may serve as a particularly important antioxidant for flying birds as they contend with variation in both reactive species production and the capacity of their antioxidant system.

Our review of the evidence to date suggests that in preparation for a migratory flight (1a) migratory birds increase some component(s) of their antioxidant capacity (notably, non-enzymatic antioxidant capacity and circulating uric acid) as they build fat stores, although increasing fat may come at the cost of increased oxidative damage; and (1b) the extent of oxidative damage associated with the increase in fat stores may be related to the extent to which birds simultaneously build antioxidant capacity in preparation for a migratory flight. (2) The acute effects of long-duration flight often include an increase in lipid damage (d-ROMs and MDA), although not in all cases, and an increase in markers of protein damage (protein carbonyls) in all cases thus far documented. Importantly, few studies document how the many components of antioxidant capacity (enzymatic, non-enzymatic, and dietary; **Figure 1**) respond during a given flight in relation to oxidative damage. Evidence to date suggests that enzymatic antioxidants, particularly GPx, as well as uric acid, a non-enzymatic endogenous antioxidant (**Figure 2**), rapidly increase in response to flight or onset of migration state. Importantly, the extent of such increases can be tissue-specific (GPx) or depend on availability of dietary antioxidants which suggests that the costs of enzymatic antioxidants and protein catabolism may be reduced when dietary antioxidants are available. Many more studies are needed that focus on the acute effects of flight before we adequately understand how such a dynamic antioxidant system responds to a host of ecologically relevant factors, including extent of flight and diet. (3) Migratory birds seem able to recover from oxidative damage accrued during long-duration flights while also rebuilding antioxidant capacity, although the extent of recovery seems to depend on bird condition (i.e., quantity and quality of a bird's remaining fat stores), and the available resources at a stopover site, including dietary antioxidants.

Given the dynamic and responsive nature of the antioxidant system, we maintain that integrative studies (from mitochondria to cells to tissues to whole organism) that are also comparative (e.g., multiple tissues within the same individuals, migration-state vs. non-migration periods, multiple species that differ in migration strategy) are required to fully understand the antioxidant system of migratory birds within an ecological context. Below we outline some future studies that would improve our understanding of this dynamic physiological system.

Intraindividual Variation Revealed by Longitudinal Studies

The ability to use endogenous and exogenous antioxidants varies among individuals depending on body condition, recent oxidative challenges, and ecological factors (Dick and Guglielmo, 2019; Loughland and Seebacher, 2020; Cooper-Mullin et al., 2021). This individual variation extends to the specific antioxidant strategies available to birds during oxidative challenges, as individuals may also rely on alternate antioxidants to protect against damage (DeMoranville, 2020). Future longitudinal studies that examine intraindividual antioxidant responses to oxidative challenges (e.g., flight, high PUFA diets, lack of dietary antioxidants, breeding, and temperature acclimatization) will help us understand the protective strategies available to birds during specific life history stages. Especially informative would be longitudinal studies that compare tissues that vary in metabolic rate, mitochondrial density, and potentially in their capacity to maintain oxidative balance (e.g., DeMoranville et al., 2020, 2021; Frawley et al., 2021).

Preparation for Migration

More controlled captive-bird experiments as well as field studies are needed that test the proposed hypothesis that as birds build fat stores in preparation for migration they also build certain components of their antioxidant capacity. More specifically, does the extent of the building of antioxidant capacity during preparation for migration *determine* the extent of observed oxidative damage. For example, to date there has been no longitudinal study of wild songbirds to parallel the multi-season assessment of Gutiérrez et al. (2019) who sampled Hudsonian Godwits at three time periods from January to March (wintering, fuelling, and pre-departure), the latter as they prepared for their >10,000-km northbound migratory flight. Marasco et al.'s (2021) study of captive-reared migratory Common Quails reminds us that controlled experiments using photoperiod manipulations to induce migration state in birds can provide important complementary (and at times contradictory) information about how the antioxidant system changes during preparation for migration. Tracking the oxidative and metabolic status of songbirds in the months and weeks leading up to departure would be particularly valuable to understanding *in situ* preparation, especially in the context of available dietary fat and antioxidant resources.

Tradeoffs: Oxidative Damage, Antioxidant Responses, and Carryover Effects

It remains unknown what amount of damage is considered *too much* damage. Our understanding of the varying response of songbirds to oxidative stress in migration and other contexts would be greatly supported by a more robust understanding of the phenomenon of oxidative damage. In particular, the time course over which the processes of RS production, antioxidant upregulation/mobilization, and RS neutralization occur during flight remains unknown. One way to evaluate this conundrum is to conduct studies that link oxidative status (antioxidant capacity and oxidative damage) to measures of short- and long-term performance (e.g., flight efficiency, speed and timing of migration, foraging, refueling, and fecundity). Doing so within the context of important life history stages such as migration and breeding would reveal potential carryover effects (McWilliams et al., 2021). Only one study to date (Skrip et al., 2016) has examined how antioxidant status during repeated flights change antioxidant allocation during subsequent breeding. At least in that model species (Zebra finch), use of dietary antioxidants to maintain oxidative balance during repeated twice-daily flights over months affected female allocation of dietary antioxidants to eggs. No studies to date of a migratory bird have determined the consequences of such carryover effects (with dietary antioxidants serving as the currency) from one stage of the annual cycle to the next on reproductive success.

Integrated Physiological Systems

There is a considerable amount of crosstalk among major physiological pathways including those involved in metabolism (PPARs, glucocorticoid production), immunity, inflammation (NF-κB), and antioxidants (NRF2 and PPARs) due to shared/multifunctional transcription factors that respond to similar factors like exercise, RS production, and dietary antioxidants (Finck and Kelly, 2006; Syeda et al., 2019). One such recent study examined these shared transcription factors and their downstream genes in a migratory bird and found that flying over the course of 2 weeks and consuming antioxidants influenced both metabolic and antioxidant mRNA expression in a tissue specific manner (DeMoranville et al., 2020, 2021). Studies that integrate across multiple systems including the metabolic, antioxidant, immune, inflammatory, endocrine, will help assess the overall state of the birds and determine if modulating the antioxidant system, for example, leads to tradeoffs with other physiological states. One such recent study of this type suggests a possible trade-off between the immune and antioxidant systems in a migratory bird (Eikenaar et al., 2018).

How Dietary Antioxidants Work

No study to date has examined the extent to which dietary antioxidants are directly used to protect accumulating fat stores and/or contribute to recovery from oxidative damage incurred during flight. The recent study by Cooper-Mullin et al. (2021) used stable-isotope labeled Vitamin E to demonstrate for the first time that consumed dietary antioxidants are absorbed and transported to the mitochondria of flight muscle, but only if birds are exercising. What remains to be demonstrated is *how* these and other types of dietary antioxidants are used during metabolism, and the extent to which they reduce the need to upregulate more costly components of the antioxidant system such as enzymes. Metabolism of all nutrients starts with processing in and absorption through the gut, and this is influenced by the gut microbiome (Clark and Mach, 2017). The extent to which variation in the gut microbiome of birds while in migration affects the availability and utilization of ingested antioxidants is not known, although such information has important implications for both our understanding of how birds work (i.e., their physiology) and also how to ensure that conservation and management efforts provide what they require during migration (Bahrndorff et al., 2016; Trevelline et al., 2019).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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A Scaling Approach to Understand the Dynamics of Fat and Lean Mass in Refueling Migrant Songbirds Measured by Quantitative Magnetic Resonance

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Fat contributes most of the energy for migratory flight of birds, whereas lean body tissues (muscles and organs) contribute amino acids and water to maintain metabolic and osmotic homeostasis. During refueling at stopover sites, both fat and lean mass are recovered, but the dynamics of this recovery are poorly understood. We used non-invasive quantitative magnetic resonance (QMR) analysis to measure fat and lean mass of > 3,500 individuals of 25 songbird species during six spring and three autumn migration seasons between 2009 and 2019 at Long Point, ON, Canada. We used allometric scaling analysis and linear mixed-effects modeling of body composition data at both the population level (single capture) and the individual level (recapture). In the population-level analysis, lean mass scaled hypoallometrically with body mass, such that for every 20% increase in body mass, lean mass was predicted to increase by 12.1% in spring and 12.8% in autumn. Fat scaled hyperallometrically with body mass, such that for every 20% increase in body mass, fat mass was predicted to increase by 144% in spring and 136% in autumn. At the individual level, these allometric relationships were more extreme. As a result of this differential allometry, at low body masses, lean and fat mass contributes nearly equally to changes in mass, but at high body mass fat deposition becomes progressively more dominant. Spring migrants deposited relatively more fat than autumn migrants, and in autumn juvenile birds tended to have greater lean mass than adults. Our findings show that lean mass deposition during refueling by songbirds is substantial, and in line with the losses of protein expected in flight. The process of fat and lean mass deposition is characterized by non-linear dynamics which are influenced by the current body composition, season, and, to a lesser extent, age. The patterns suggest that the need for dietary protein to rebuild lean mass will be greater when body mass is low, during autumn migration, and in juvenile birds.

Keywords: body composition, allometry, migration, bird, energetics, nutrition

INTRODUCTION

Knowledge of the changes in body composition that occur at staging and stopover sites is essential to our understanding of how migratory birds budget fuel, and of the nutrients they require when fueling. Early work on fuel storage of migratory birds produced the airplane paradigm where lean body components stayed constant, and fat was used as fuel for flight (Connell et al., 1960; Odum et al., 1964). It was later recognized that although stored fat provides the majority of energy for migratory flight, the catabolism of lean body components during flight and their anabolism (i.e., growth) during fueling is substantial and widespread among migratory birds (Piersma and Jukema, 1990; Lindström and Piersma, 1993; Klaassen et al., 1997; Bauchinger and Biebach, 1998; Jenni and Jenni-Eiermann, 1998; Karasov and Pinshow, 1998; Schwilch et al., 2002). Some of the most dramatic examples come from situations where birds prepare to cross, or have just finished crossing, large ecological barriers such as the Sahara Desert or Pacific Ocean. For example, when Garden Warblers (*Sylvia borin*) migrate across the Sahara, 70% of mass lost is fat and 30% is lean tissue protein from muscles and organs (Biebach, 1998). Trans-Pacific migrant shorebirds show extreme changes in both fat and lean body components as they prepare for and undertake multi-day flights (Piersma and Gill, 1998; Battley et al., 2000). However, the limited evidence available indicates that even songbirds refueling at stopovers where there is no ecological barrier appear to deposit significant amounts of lean mass along with fat (Karasov and Pinshow, 1998; Seewagen and Guglielmo, 2011; Wojciechowski et al., 2014). Unlike body fat, which can be synthesized from dietary fat, carbohydrate, or protein, the deposition and maintenance of lean mass stores require dietary protein intake. Thus, factors that influence lean mass deposition could influence diet selection for protein-rich foods, such as invertebrates, during refueling (Carpenter et al., 1993).

The composition of fuel deposited at stopover is related to the fuel mixture in flight. Wind tunnel studies have confirmed that lean mass (protein) provides about 5–10% of the energy for endurance flight of birds (Klaassen et al., 2000; Jenni-Eiermann et al., 2002; Gerson and Guglielmo, 2011; Guglielmo et al., 2017; Dick and Guglielmo, 2019; Groom et al., 2019). Catabolism of protein occurs as a result of normal protein turnover, to maintain stable blood glucose concentration, to provide key intermediates of the citric acid cycle, and to provide water under dehydrating conditions (Klaassen, 1996; Bauchinger and Biebach, 1998; Jenni and Jenni-Eiermann, 1998; Gerson and Guglielmo, 2011; Groom et al., 2019). It may also confer an adaptive advantage for long flights by reducing excess mass, and thus power costs for flying (Pennycuick, 2008). Because wet lean tissue has a much lower energy density than fat (5.4 vs. 37.6 kJ g^{-1} wet; Jenni and Jenni-Eiermann, 1998), the small contribution of protein to flight energy results in a proportionally large loss of mass, which has been estimated to be 30 and 47% of total mass loss for a 5 and 10% contribution to energy, respectively (Salewski et al., 2009). Thus, it may be expected that lean mass deposition at refueling sites will be in the same range as birds prepare for the next flight.

Along with recognizing the importance of lean mass dynamics, some previous studies have argued that the body lean and fat masses can be partitioned into components associated with structural size (Piersma and Davidson, 1991), some of which may be used as "reserves" in extreme situations, and "stores" which are deposited above the basic structure in anticipation of activities like enduring periods of food shortage, surviving a cold winter night, migration, or reproduction (van der Meer and Piersma, 1994). Under the assumption that there is a distinct difference between reserves and stores, authors interested in the composition of stores have attempted to estimate and subtract off the structural component (e.g., mass of birds with zero visible fat; Salewski et al., 2009), or have fitted breakpoint regression models to identify an abrupt change at the threshold of stores and reserves (van der Meer and Piersma, 1994). The latter is based on the physiological transition in fuel use between phase II and phase III of fasting in long-term starved birds (Le Mayo et al., 1981), which may not be equivalent to the physiology of fuel deposition, and may not be relevant for songbirds at stopover. Alternatively, others have fit breakpoints in lean and fat mass at high body masses where an assumed threshold of maximum lean mass had been attained (biphasic mass gain; Carpenter et al., 1993; Wojciechowski et al., 2014), while others have reported that fuel deposition may even occur in three phases in shorebirds preparing for a long barrier crossing (Piersma et al., 1999; Hua et al., 2013).

An important point raised by Lindström and Piersma (1993), in reference to population-level analyses of body composition (i.e., one sample per individual), was that the relative deposition of lean mass in stores could be overestimated by regression of lean mass on body mass due to a poor accounting of how structural mass increases with structural size. Instead, they suggested that reliable estimates of lean and fat deposition required sampling synchronously migrating populations through time as they refueled at stopover. They were only able to apply this method to shorebirds and waterfowl because they did not find examples of synchronously arriving and departing songbirds, but they anticipated a future where non-invasive technologies would allow repeated measurements of recaptured individuals as they refueled. Such methods are now available.

Quantitative magnetic resonance (QMR) allows quick, safe, and accurate repeated measurement of fat and lean mass of awake birds (Guglielmo et al., 2011; Kennedy et al., 2016; Kelsey et al., 2019). This technology has been validated on a variety of taxa including small animals like mice, rats, bats, and birds (Taicher et al., 2003; McGuire and Guglielmo, 2010; Guglielmo et al., 2011). QMR predicts the masses of fat, wet lean, and total body water of small birds with precisions of $< \pm 3\%$ and accuracies of approximately $\pm$ 11, $\pm$ 2 and $\pm$ 2%, respectively (Guglielmo et al., 2011). Seewagen and Guglielmo (2011) used QMR to measure the deposition of fat and lean mass in migrant passerines at a stopover site and showed that on average 35% of the total mass gained in four individual recaptured birds was lean mass. At the population level, they also used regression analysis of body composition of single captured birds and estimated lean mass contributions of similar magnitude in four species (Seewagen and Guglielmo, 2011). Kelsey and Bairlein (2019) used

QMR to measure body composition changes during migratory fueling of captive wheatears (*Oenanthe oenanthe*) (Kelsey and Bairlein, 2019). Kelsey et al. (2019, 2021) used QMR to derive predictive equations of lean mass and fuel load of birds at stopover, and to test for differences in lean mass relative to size in bird species with different migration strategies (Kelsey et al., 2019, 2021). We have used QMR in a variety of contexts to study fuel use in flight (Gerson and Guglielmo, 2011; Guglielmo et al., 2017; Dick and Guglielmo, 2019) and the influence of fat and lean mass on migration behavior (Brown et al., 2014; Kennedy et al., 2016; Morbey et al., 2018; Beauchamp et al., 2020). Other non-invasive methods include dual x-ray absorptiometry (DEXA) (Korine et al., 2004; Wojciechowski et al., 2014), heavy water dilution (Ellis and Jehl, 1991; Karasov and Pinshow, 1998; McWilliams and Whitman, 2013), and total body electrical conductivity (TOBEC) (Karasov and Pinshow, 1998; McWilliams and Whitman, 2013). DEXA requires anesthesia and X-ray safety precautions, while TOBEC is no longer commercially available. Heavy water dilution is relatively easy, practical, and inexpensive, but requires holding birds for about 15–30 min and usually requires taking body fluid samples (e.g., blood, but urine is a suitable alternative). It can be made even less invasive if breath deuterium enrichment is measured in real time with laser spectroscopy (Mitchell et al., 2015).

In contrast to accounting for structural mass when exploring the composition of stores, a more fundamental approach is to statistically model the scaling relationships of lean and fat mass with body size and body mass. Scaling relationships describe how body parts or components change in size with a change in overall size (Schmidt-Nielsen, 1984). Isometric scaling means that a component changes in direct proportion (i.e., linearly) to body size. Hypoallometric scaling means that the component increases with body size, but at less than proportionality. Under hyperallometric scaling, the component increases in both absolute size and relative proportion with body size. This phenomenological approach is simple, avoids making assumptions about the underlying physiology, and has the potential to describe non-linearities that approximate breakpoint-like phenomena. Moreover, scaling analyses can be applied across species and contexts to derive generalities and test for the effects of covariates of interest.

Although lean mass deposition may be widespread among migratory birds, covariates or factors that determine variation in the relative deposition of fat and lean mass are very poorly understood. Factors such as species, season, sex, age, or migration strategy may affect the mixture of fuels deposited. Species could differ in the relative deposition of lean and fat mass at stopover due to differences in diet (Gannes, 2001), body size (Lindström and Piersma, 1993), or migration distance (Kelsey et al., 2021). Seasonal differences in fuel accumulation may be attributed to different ultimate goals between spring (reproduction) and autumn (overwintering), or because food and weather conditions are more unpredictable in spring (Newton, 2008). There is some evidence that body mass and fat stores are greater in the spring (Seewagen and Slayton, 2008; Kennedy, 2012; Schmaljohann et al., 2018), but there is little information on seasonal variability in lean mass (Salewski et al., 2009; Wojciechowski et al., 2014).

Age could affect fat and lean mass accumulation, particularly during autumn. Passerine birds generally have low survival rates in their first year of life, and mortality may be especially great during migration (Sillet and Holmes, 2002; McKim-Louder et al., 2013). The energy demands of migration may be greater for juveniles since they may have greater maintenance metabolic costs than adults (McCabe and Guglielmo, 2019), convert less of ingested energy to stores (McCabe and Guglielmo, 2019), and tend to struggle more to maintain stored fuel relative to adults (Swanson et al., 1999). Hatch year birds in fall may be poorer foragers (Heise and Moore, 2003; Vanderhoff and Eason, 2007), may choose poorer quality foods, or may be out-competed for food by adults (Sol et al., 1998). Differences in gut size and continued maturation in hatch year birds could also lead to differences in lean mass dynamics, particularly in the gut (Hume and Biebach, 1996; Guglielmo and Williams, 2003; McCabe and Guglielmo, 2019).

We used QMR to measure body composition of a variety of songbird species at a single site during 6 spring and 3 autumn migrations over a 10-year period. Here we use a scaling approach of population-level (cross-sectional) and individual-level (recapture) data to analyze the composition of fuel accumulated at stopover. We use linear mixed modeling to examine general effects of season, age, and migration distance while allowing for random effects of species and year. Based on previous studies and the known contribution of lean mass to energy in flight, we hypothesized that both fat and lean mass change during refueling, with lean mass accounting for about 30–50% of mass gain. We also tested for non-linearity in the deposition of fat and lean mass. We predicted that spring migrants would accumulate a greater proportion of fat than lean mass and that juvenile birds would accumulate less fat relative to adults. Finally, we sought to assess the congruence between individual- and population-level analyses. If individual-level assessment supported population-level analysis, then future studies on migrant refueling would not need to rely on recaptures, which are less common and may be biased toward birds that remain at stopover longer.

MATERIALS AND METHODS

Study Site and Data Collection

The research procedures were approved by the University of Western Ontario Animal Care Committee (protocols 2006-014, 2010-020, 2017-160) and permitted by the Canadian Wildlife Service (Migratory Bird Banding Permit 10169). A variety of songbirds were sampled at the Old Cut study site of the Long Point Bird Observatory (LPBO) on Long Point, ON, Canada (42°34′57.71″N, 80°23′51.48″W) in six spring and three autumn migration periods between 2009 and 2019 for several different research projects (Kennedy, 2012; Brown et al., 2014; Morbey et al., 2018; Beauchamp et al., 2020; Deakin et al., unpublished data). Spring data were collected as early as April 25 and as late as May 26, whereas autumn data were collected as early as August 27 and as late as October 21. Each day 14 mist-nets in a woodlot were opened 30 min before sunrise and remained open for 6 h. Nets were not open during periods of heavy rain or wind. Birds were individually banded with a unique nine-digit number on a Canadian Wildlife Service/United States Fish and Wildlife Service aluminum band. For each bird, LPBO staff recorded species, age, sex, unflattened wing chord (mm), mass (0.1 g), visible fat (scored on a scale of 0–7) (LPBO, 2019), date, and time of capture. Sex and age were determined by species-specific plumage and molt criteria, eye and/or tongue coloration, and degree of skull pneumatization for age during autumn (Pyle, 1997). All spring birds were considered to be adults. In autumn, birds were classified as adult (i.e., after-hatch-year) or juvenile (i.e., hatch-year).

Birds were then brought to a mobile laboratory at the site to be scanned in the QMR body composition analyzer (Echo-MRI-B, Echo Medical Systems, Houston, TX, United States), which measures dry fat and wet lean mass of small birds with high precision (CV of 2.4 and 0.7% for fat and wet lean mass, respectively) and accuracy (relative errors of 6–11% for fat and 1–2% for wet lean mass) (Guglielmo et al., 2011). Birds were scanned once using the "small bird" (< 50 g) and "two-accumulation" settings as described in Guglielmo et al. (2011). Scanning once decreases precision and thus increases experimental error, but allows one to scan many more species and individuals. In this study, the maximum number of birds scanned in a 6 h period was 140. The QMR was calibrated using a 94 g canola oil standard provided by the manufacturer. A daily "system test" was done with the 94 g oil standard and, depending on the year of study, scans of 3, 5, and/or 10 g canola oil standards were taken periodically throughout the day to ensure scanning accuracy. Each bird was held in a ventilated plexi-glass tube appropriate for its size for a 1.5–2 min scan. Birds were selected for scanning after banding only if there was sufficient time for processing since no bird was held after capture for more than 1 h. All birds were released immediately after scanning. Fat mass and wet lean body mass values were adjusted using two calibration equations (calibrated fat mass = raw QMR fat x 0.94, and calibrated lean body mass = raw QMR wet lean body mass x 1.021) derived from a laboratory validation developed from house sparrows (*Passer domesticus*) and zebra finches (*Taeniopygia guttata*) (Guglielmo et al., 2011).

Cross-Sectional Analysis of Single Quantitative Magnetic Resonance Scans

For the cross-sectional analysis of single scan data (not recaptures), only data for initial scans were included. Data were first filtered to include all species with at least 20 unique individuals with complete information for wing chord, mass, fat mass, and lean mass. Outliers, which were attributed to transcription errors, were detected and removed following standard procedures (Zuur et al., 2010). We removed observations with fat ≤ 0 g ($n = 20$) as these values were unrealistic and could not be $\log_{10}$-transformed. By species, we removed points outside of the 99% confidence interval for mass and wing chord. We also removed large residuals (absolute value > 0.04) in the $\log_{10}$(mass) vs. $\log_{10}$(fat mass + lean mass) regression ($n = 67$). If any year-season-species combination fell below a sample size of 20, this combination was removed from the dataset. We also removed female Red-winged Blackbirds (*Agelaius phoeniceus*, $n = 5$), as they showed different scaling relationship than males. The number of species sampled per season and year is shown in **Figure 1**, and included 3,549 observations (**Table 1**).

We used a linear mixed modeling approach to summarize average scaling relationships for species stopping over at Long Point, where a scaling relationship follows this form:

$$y_i = \alpha {x_i}^{\beta}$$

$$\log_{10}(y_i) = \log_{10}(\alpha) + \beta \log_{10}(x_i)$$

For scaling relationships between body mass, lean mass, and fat mass with structural size, we used wing chord as an index of structural size. The only other structural size metric available was tarsus length, but this was not always measured and was less correlated with body mass within species. In models, mass and wing chord were $\log_{10}$-transformed, which is the convention for scaling studies, and the slope represents the scaling exponent β. Because wing chord is a linear measurement and mass is a volumetric measurement, isometry would be indicated by mass scaling with the cube of wing chord. Thus, when interpreting β for scaling with wing chord, we classified $\beta < 3$ as hypoallometric, $\beta = 3$ as isometric, and $\beta > 3$ as hyperallometric.

In the scaling models, we specified random effects for species ($n = 25$) and year-season combinations ($n = 9$). This allowed us to account for variation in the scaling relationships among distinct species and sampling seasons and permitted us to obtain the expected scaling relationship for a typical bird at this stopover location. The compromise is that group-level slopes show shrinkage (i.e., are less extreme) relative to group-level linear model estimates (Hector, 2015). For species random effects, we selected between random intercept and random intercept and slope models using likelihood ratio tests (LRTs). We retained random intercepts for the year-season combination because this was an essential component of the sampling scheme. Sex and age class were excluded from the global model as they were sampled unevenly across species, but exploratory analyses showed consistent scaling relationships regardless of sex or age class (except for the aforementioned Red-winged Blackbird). For all linear mixed models, we were interested in the variance components, the fixed effects, and the marginal and conditional R^2-values (Nakagawa and Schielzeth, 2013), which are the proportion of variance explained by fixed and fixed + random effects, respectively. In R version 4.0.0 (R Core Team, 2020), we used function lmer in package lme4 to fit linear mixed models (Bates et al., 2015), and r.squaredGLMM in MuMIn for R^2-values (Barton, 2020). Model fit and homogeneity of variance were confirmed through visual inspection of residuals.

For species with sufficient sample sizes for evaluation of functional relationships ($n > 60$), we used standardized major axis (SMA) regression to estimate β in the bivariate relationships between each mass component and wing chord. As opposed to ordinary least squares regression, SMA regression minimizes the area formed by the right triangles between the point and regression line (Warton et al., 2006). SMA was selected over

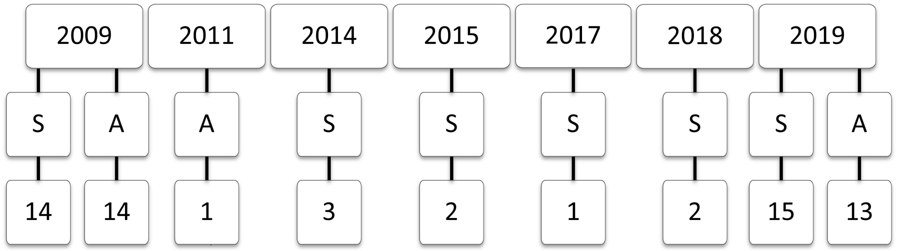

FIGURE 1 | The number of songbird species with $n \geq 20$ individuals sampled across seasons (S, spring; A,autumn) in 2009 (Kennedy, 2012), 2011 (Brown et al., 2014), 2014 and 2015 (Morbey et al., 2018), 2017 (Beauchamp et al., 2020), and 2018 and 2019 (Deakin et al., unpublished data).

TABLE 1 | Songbird species used in the study of lean and fat mass scaling, showing the species code, scientific name, common name, single scan sample size, migration distance, and breeding location (Carolinian Zone or not).

Code	Scientific name	Common name	*n*	Distance[a]	Carolinian
RWBL	*Agelaius phoeniceus*	Red-winged Blackbird	32	Short	1
CAWA	*Cardellina canadensis*	Canada Warbler	22	Long	0
HETH	*Catharus guttatus*	Hermit Thrush	154	Short	0
GCTH	*Catharus minimus*	Gray-cheeked Thrush	26	Long	0
SWTH	*Catharus ustulatus*	Swainson's Thrush	203	Medium	0
GRCA	*Dumetella carolinensis*	Gray Catbird	175	Medium	1
LEFL	*Empidonax minimus*	Least Flycatcher	35	medMex	0
COYE	*Geothlypis trichas*	Common Yellowthroat	132	medium	1
BAOR	*Icterus galbula*	Baltimore Oriole	30	medMex	1
NAWA	*Leiothlypis ruficapilla*	Nashville Warbler	48	medMex	0
SWSP	*Melospiza georgiana*	Swamp Sparrow	26	Short	1
SOSP	*Melospiza melodia*	Song Sparrow	24	Short	1
RBGR	*Pheucticus ludovicianus*	Rose-breasted Grosbeak	24	medMex	1
RCKI	*Regulus calendula*	Ruby-crowned Kinglet	314	Short	0
GCKI	*Regulus satrapa*	Golden-crowned Kinglet	112	Short	0
OVEN	*Seiurus aurocapilla*	Ovenbird	30	Medium	1
BTBW	*Setophaga caerulescens*	Black-throated Blue Warbler	127	Medium	0
MYWA	*Setophaga coronata*	Myrtle Warbler	126	Short	0
MAWA	*Setophaga magnolia*	Magnolia Warbler	440	Medium	0
YWAR	*Setophaga petechia*	Yellow Warbler	85	Medium	1
AMRE	*Setophaga ruticilla*	American Redstart	235	Medium	1
HOWR	*Troglodytes aedon*	House Wren	63	Short	1
REVI	*Vireo olivaceus*	Red-eyed Vireo	25	Long	1
WTSP	*Zonotrichia albicollis*	White-throated Sparrow	1,037	Short	0
WCSP	*Zonotrichia leucophrys*	White-crowned Sparrow	24	Short	0

[a] *Short, winter in the southern United States; medium, winter among coastal regions or islands in the Caribbean subtropics; medMex, winter in Mexico; long, winter in South America.*

major axis regression as the former is appropriate when variables have different units. However, SMA regression was not done for weak bivariate relationships, as this contraindicates a true functional relationship.

We next examined scaling relationships between the fat and lean components of body mass and total body mass. Again, the component masses and body mass were $\log_{10}$-transformed, and the slope represents the scaling exponent β. Now when interpreting β, we classified $\beta < 1$ as hypoallometric, $\beta = 1$ as isometric, and $\beta > 1$ as hyperallometric. In models, we specified random effects for species and year-season combinations. For random effects for species, we selected between random intercept and random intercept and slope models using LRTs. We retained random intercepts for the year-season combination. Sex and age class were excluded from the global model. However, we included season and the mass-by-season interaction, as we were specifically interested in comparing scaling relationships between spring and autumn. To interpret how the scaling coefficients (βs) would translate into a refueling bird, we predicted the effect of a 20% increase in body mass, calculated as $(1.2^{\beta}-1)*100\%$, which applies regardless of body mass. We also predicted the effect of an absolute +1 g difference in body mass from the median body mass for each species, but caution that these effects cannot be extrapolated because we are modeling multiplicative, not additive, effects.

We determined whether there was support to include age (autumn only), and migration distance as fixed effects in the models of body composition using LRTs. For age comparisons in autumn, species with fewer than 20 observations of each age class (adults, juvenile) were excluded. We considered two aspects of the migration distance. Based on the Birds of the World (Billerman et al., 2020), we assigned species as short distance (winter in the southern United States), Caribbean medium distance (winter among coastal regions or islands in the subtropics), Mexico medium distance (winter in Mexico), or long-distance migrants (winter in South America). We also assigned breeding region as a binary variable based on whether the species breeds at high density in the Carolinian region of Ontario (where our study site was located) or further north based on the Atlas of the Breeding Birds of Ontario (Cadman et al., 2007).

In the mixed-effects models, species were assumed to be independent even though species with shared ancestry may be more similar in their response variables. We considered using phylogenetically controlled analyses, which typically use species-level data. However, with our individual-level data, it was not straightforward to incorporate phylogenetic similarity as random intercepts and slopes, while also allowing for random intercepts for year-season combinations. To partially address the issue of phylogenetic similarity, we explored structure in the random slope estimates for species with and without controlling for phylogeny (for methods, see **Supplementary Material**).

Longitudinal Analysis of Recaptures

To evaluate body composition change within individuals over time while at the stopover site, we selected all birds that were recaptured and scanned on two or more occasions on different days less than 8 days apart, and then selected the initial and final scans. We compared the change in body mass measured gravimetrically by a balance to the change in body mass (fat + wet lean mass) measured by the QMR (M_q), and removed 6 large residuals (<-1 or > 1). The final data included 145 recaptures across 25 species, with 52 recaptures for White-throated Sparrows and 1–11 recaptures in each of the remaining species. Change in M_q was strongly correlated with change in body mass ($R = 0.966$; OLS intercept –0.014 [CI: –0.066, 0.037], slope 0.878 [CI: 0.839, 0.917]). We calculated percent fat mass change and percent lean mass change and determined how these related to percent body mass change using general linear models. Given the allometric relationships between the body mass components and body mass determined from single scan analysis, we expected these relationships to be linear. We tested for the inclusion of season and species grouping (White-throated Sparrows *Zonotrichia albicollis* vs. all other species) using LRTs. Species grouping was considered because the dataset was dominated by recaptures of White-throated Sparrows.

Predictive Equations

To facilitate prediction of energy stores in migrants we constructed species-specific predictive equations for fat (g) as a function of body mass, wing chord, and fat score. Data for both first captures and recaptures were used ($n = 4{,}016$), which increased the number of species with ≥ 20 samples from 25 to 32. Due to collinearity among variables, we used LASSO regression to lessen the problem of high variance and to allow for the elimination of highly correlated predictors (Dormann et al., 2013). This was done using functions in glmnet (Friedman et al., 2010). We also ran these models using half of each dataset as the training set, and the other half as the test dataset. The predicted R^2 provided an index for how well the training model predicted fat in the test data. Other linear model constructions were considered, including log-transformations and use of the scaled mass index (Peig and Green, 2009) instead of including mass and wing chord separately, but these did not perform better than the simple linear equations. We did not attempt to predict lean mass stores because the data lacked an index of lean mass, such as a muscle score (Salewski et al., 2009).

RESULTS

Scaling With Structural Size

In the pooled dataset, body mass and wing chord were strongly, positively correlated ($R = 0.857$, $P < 0.0001$, $n = 3{,}549$). In the analysis of body mass scaling, there was evidence to support a random intercept and slope model over a random intercept only model (LRT: $\chi^2 = 8.3$, df = 2, $P = 0.016$), and the random effects accounted for more of the variance (87.0%) than the fixed effect of wing chord (11.0%). Overall, the intraspecific body mass scaling coefficient (i.e., the fixed effect of wing chord) was 0.906 (CI: 0.793, 1.036), which indicates hypoallometry (**Figure 2A**). Among the random effects, species effects contributed much more than year-season effects (**Table 2**). Among species, the random slope estimates increased with wing length, with or without controlling for phylogeny (**Supplementary Material**). This suggests that $\log_{10}$-transformation did not completely linearize the relationship between body mass and wing chord. However, variation in the random slopes was subtle compared to the global body mass scaling coefficient. Among the 13 species with $n > 60$ observations, the mean SMA slope was 2.18; 10 of 13 species showed hypoallometry of body mass with wing chord with SMA slopes < 3, and three species showed isometry with SMA slopes not different from 3.0 (**Table 3**).

Lean mass and wing chord were also strongly, positively correlated ($R = 0.865$, $P < 0.0001$, $n = 3{,}549$). In the analysis of lean mass scaling, there was strong evidence to support a random intercept and slope model over a random intercept only model (LRT: $\chi^2 = 38.7$, df = 2, $P < 0.0001$), and the random effects accounted for more of the variance (89.9%) than the fixed effect of wing chord (9.0%). Overall, the intraspecific lean mass scaling exponent was 0.878 (CI: 0.732, 1.035), which indicates hypoallometry with structural size (**Figure 2B**). Among the random effects, species effects contributed much more than year-season effects (**Table 2**). Among species, the random slope estimates varied independently of wing length (**Supplementary Material**). This suggests that $\log_{10}$-transformation effectively linearized the relationship between lean mass and wing chord.

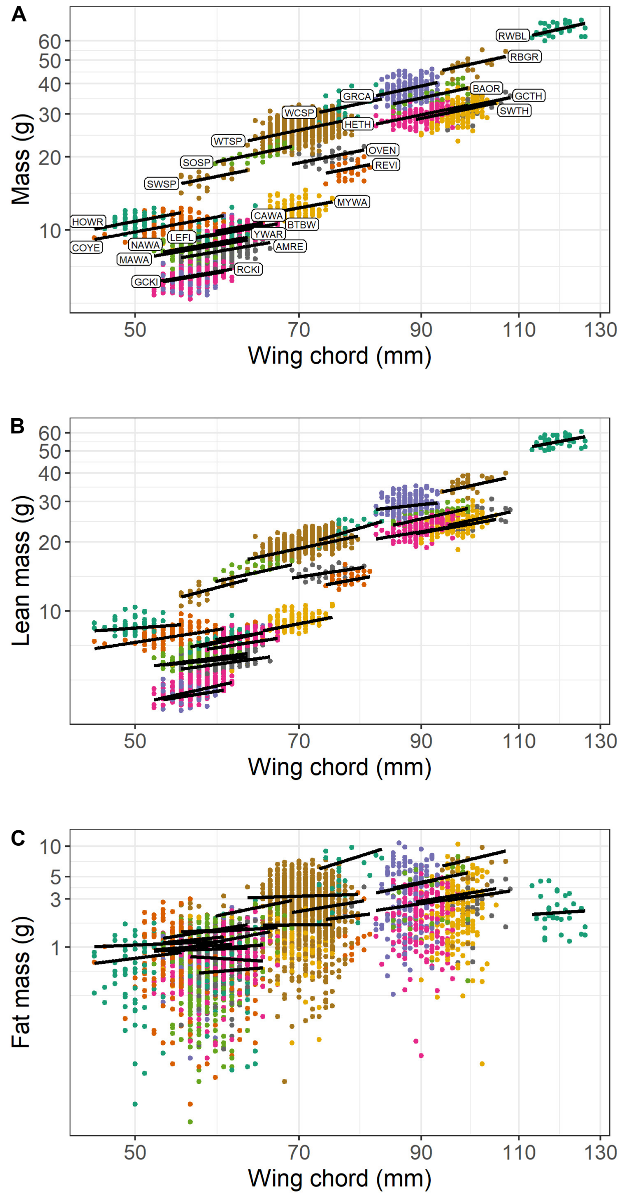

FIGURE 2 | Scaling of body mass **(A)**, lean mass **(B)**, and fat mass **(C)** with wing chord for 25 species of songbirds stopping over at Long Point, Ontario ($n = 3{,}549$). The lines show the linear mixed model predictions when year = 2009 and season = spring. Species codes are shown in panel A and defined in **Table 1**.

TABLE 2 | Random effects in scaling relationships with structural size for songbirds stopping over at Long Point, Ontario.

Scaling model	Random component	*SD*
Mass vs. wing chord		
Species ($n = 25$)	Intercept	0.284
	$\log_{10}$(wing chord)	0.190
Year:Season ($n = 9$)	Intercept	0.008
Residual		0.031
Lean mass vs. wing chord		
Species ($n = 25$)	Intercept	0.549
	$\log_{10}$(wing chord)	0.307
Year:Season ($n = 9$)	Intercept	0.009
Residual		0.025
Fat mass vs. wing chord		
Species ($n = 25$)	Intercept	2.273
	$\log_{10}$(wing chord)	1.296
Year:season ($n = 9$)	Intercept	0.109
Residual		0.260

For species, random intercepts and slopes are shown.

TABLE 3 | Exponents (with 95% confidence interval) in body mass and lean mass scaling relationships with wing chord for songbirds stopping over at Long Point, Ontario.

Species	β, Mass	β, Lean mass
AMRE	2.01 (1.79–2.26)	1.69 (1.50–1.90)
BTBW	1.92 (1.63–2.27)	1.98 (1.68–2.33)
COYE	1.88 (1.60–2.20)	1.62 (1.39–1.88)
GCKI	2.76 (2.31–3.30)	2.76 (2.31–3.30)
GRCA	3.00 (2.59–3.47)	2.69 (2.32–3.11)
HETH	1.86 (1.61–2.14)	2.05 (1.78–2.37)
HOWR	1.55 (1.21–1.98)	1.75 (1.36–2.25)
MAWA	2.10 (1.92–2.30)	1.75 (1.61–1.91)
MYWA	2.21 (1.86–2.62)	2.28 (1.96–2.66)
RCKI	2.24 (2.03–2.48)	2.36 (2.15–2.59)
SWTH	2.68 (2.37–3.04)	2.29 (2.01–2.60)
WTSP	2.12 (2.00–2.24)	1.75 (1.67–1.84)
YWAR	2.04 (1.68–2.49)	1.91 (1.58–2.31)

Estimates were based on standardized major axis regression in species with $n > 60$ observations.

Overall, random slopes were subtle compared to the global intraspecific lean mass scaling coefficient. Among the 13 species with $n > 60$ observations, the mean SMA slope was 2.07; 11 of 13 species showed hypoallometry of lean mass with wing chord with SMA slopes < 3, and two species showed isometry with SMA slopes not different from 3.0 (**Table 3**).

Fat mass and wing chord were significantly positively correlated ($R = 0.541$, $P < 0.0001$, $n = 3{,}549$), but less so than for lean mass. In the analysis of fat mass scaling, there was evidence to support a random intercept and slope model over a random intercept only model (LRT: $\chi^2 = 8.0$, df = 2, $P = 0.019$). The random effects accounted for more of the variance (45.5%) than fixed effects (7.9%), but there was also substantial unexplained variation. Overall, the intraspecific fat mass scaling exponent was 1.311 (CI: 0.527, 2.137), which indicates an overall pattern of hypoallometry with structural size (**Figure 2C**). Among the random effects, species effects contributed more than year-season effects (**Table 2**). Among species, the random slope estimates increased with wing length, and more so when accounting for phylogeny (**Supplementary Material**). This suggests that $\log_{10}$-transformation did not completely linearize the relationship between fat mass and wing chord. However, random slopes were subtle compared to the global intraspecific fat mass scaling coefficient. SMA regression was not warranted, as fat mass was not functionally related to wing chord within species.

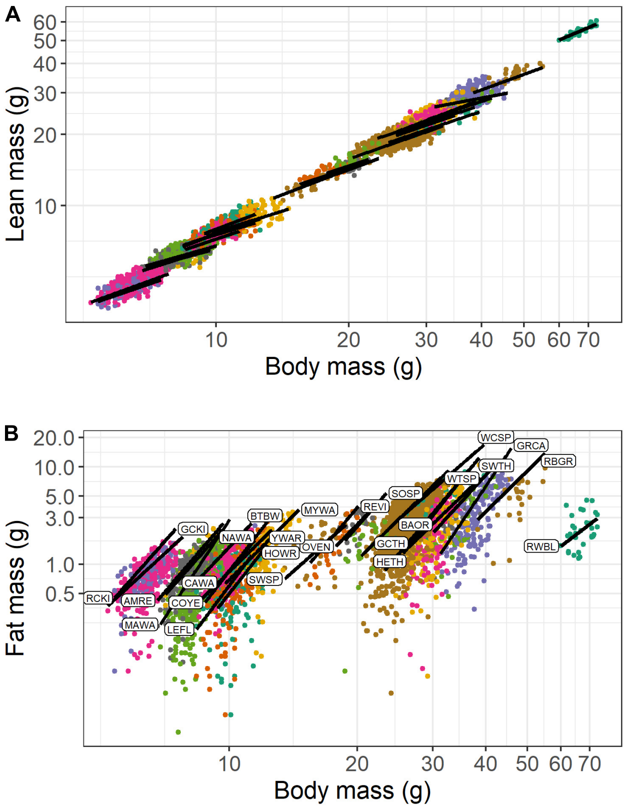

FIGURE 3 | Scaling of lean mass **(A)** and fat mass **(B)** with body mass for 25 species of songbirds stopping over at Long Point, Ontario (*n* = 3,549). The lines show the linear mixed model predictions when year = 2009 and season = spring. Species codes are shown in panel B and defined in **Table 1**.

Scaling With Body Mass and Seasonal Differences

In the analysis of lean mass scaling with body mass, there was strong evidence to support a random intercept and slope model over a random intercept only model (LRT: χ^2 = 51.4, df = 2, $P < 0.0001$). Random effects accounted for less of the variance (30.5%) than fixed effects (68.7%). Overall, the relationship between lean mass and the fixed effect of body mass was hypoallometric (**Figure 3A**), and slightly more hypoallometric in spring (β = 0.627) than in autumn (β = 0.658; difference in β = 0.032 [CI: 0.025, 0.038]). For a 20% increase in body mass, lean mass was predicted to increase by 12.1% in spring and 12.8% in autumn. In terms of absolute values, birds that differed by +1 g in body mass (from the species-specific median), were predicted to differ in lean mass by +0.260 to +0.646 g in spring (mean = +0.457 g) and +0.304 to 0.733 g in fall (mean = +0.495 g). Model predictions by season are shown for the 11 species that had sufficient spring and fall data (**Figure 4A**). The figure indicates the subtle difference in scaling between seasons and shows that birds generally had less lean mass at the same body mass in spring than in autumn. Among the random effects, species effects contributed much more than year-season effects (**Table 4**), and random slopes did not vary with mean body mass, with or without phylogenetic correction (**Supplementary Material**).

In the analysis of fat mass scaling with body mass, there was strong evidence to support a random intercept and slope model over a random intercept only model (LRT: χ^2 = 63.6, df = 2, $P < 0.0001$). Random effects accounted for less of the variance (43.0%) than fixed effects (55.7%). Overall, the relationship between fat mass and body mass was consistently hyperallometric (**Figure 3B**), and slightly more hyperallometric in spring (β = 4.89) than in autumn (β = 4.70; difference in β = 0.186 [CI: 0.120–0.252]). Model predictions by season are shown for the 11 species that had sufficient spring and autumn data (**Figure 4B**). The figure indicates the subtle difference in scaling between seasons and shows that birds generally had more fat mass at the same body mass in spring than in autumn. Among all birds, for a 20% increase in body mass, fat mass was predicted to increase by 144% in spring and 136% in autumn. Birds that differed by +1 g in body mass (from the median), differed in fat mass by +0.111 to +1.109 g (mean = +0.656 g) in spring and by +0.060 to +0.936 g (mean = +0.477) in autumn. Among the random effects, species effects contributed much more than year-season effects (**Table 4**), and random slopes did not vary with mean body mass, with or without phylogenetic correction (**Supplementary Material**).

The seasonal differences in body composition are unlikely to be caused by the autumn samples being sometimes dominated by juvenile birds (21–96%, median 84% juvenile) because age differences in body composition in autumn were very subtle (see below) compared to the seasonal differences. In addition, in the White-throated Sparrow where there were > 20 observations for adult birds in a spring and fall season of the same year (2009), the increase in lean mass in the spring was less steep than in the fall (β = −0.326, CI: −0.558, −0.095, df = 98, t = −2.80, P = 0.006), and overall lean mass was greater in the fall (β = 0.433, CI: 0.108, 0.758, df = 98, t = 2.64, P = 0.010). For fat, the relationship between $\log_{10}$(fat) and $\log_{10}$(mass) did not depend on season (t = 1.39, df = 98, P = 0.168), and after dropping the interaction, fat mass was greater in the spring than in the fall (β = 0.217, CI: 0.150, 0.283, t = 6.48, df = 99, $P < 0.0001$).

To illustrate the interaction between hypoallometry of lean mass and hyperallometry of fat, the effect of a broad range of mass change (−5 to +5 g) on predicted changes in lean mass and fat is shown for White-throated Sparrows (**Figure 5**). At low and intermediate masses fat and lean mass are deposited in nearly equal amounts, and at high body masses fat deposition exceeds lean mass deposition.

Migration Distance and Age

Compared to the linear mixed models for scaling with body mass as a fixed effect, shown above, there was some weak evidence that breeding region improved model fit for lean mass (LRT: χ^2 = 2.83, df = 1, P = 0.092) and fat mass (χ^2 = 2.46, df = 1, P = 0.117), with Carolinian species predicted to have higher lean

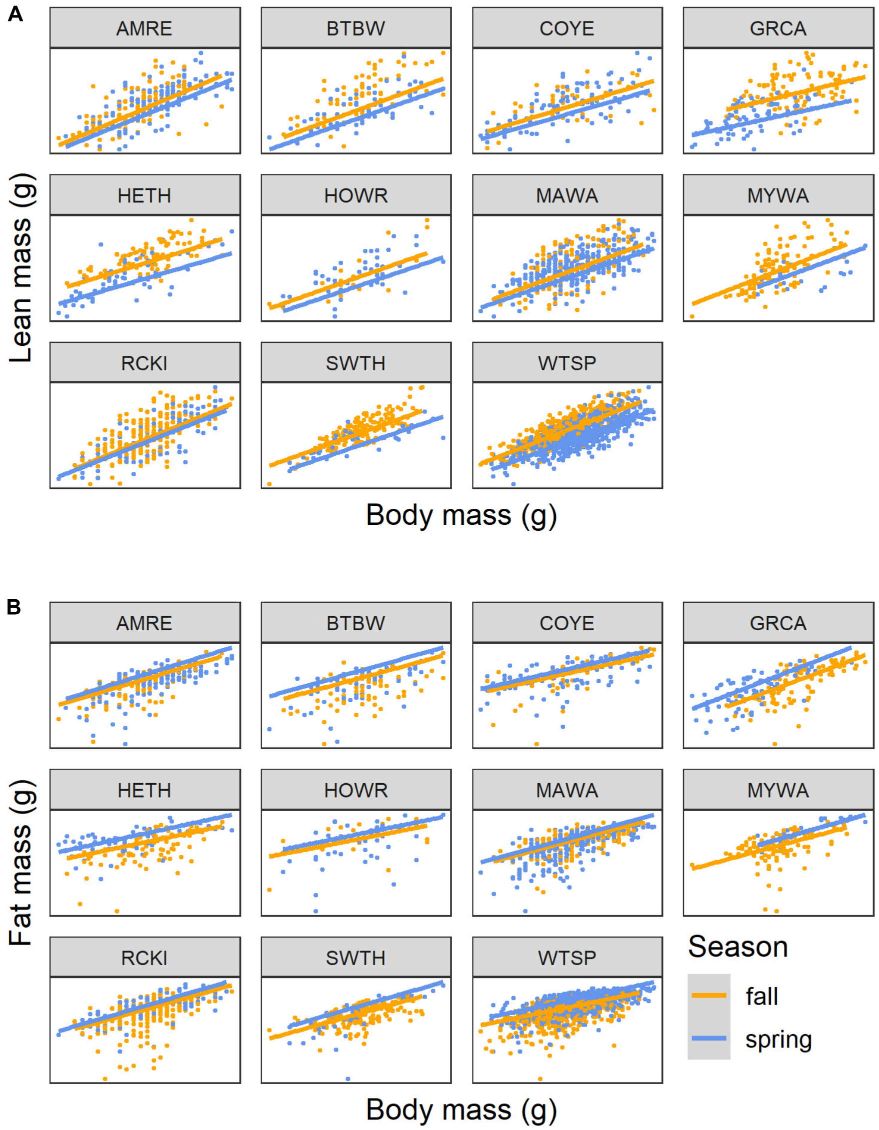

FIGURE 4 | Scaling of lean mass **(A)** and fat mass **(B)** with body mass for 11 species of songbirds stopping over at Long Point, Ontario, highlighting differences between seasons. The lines show the linear mixed model predictions for spring (blue) and autumn (orange) when year = 2009. Ranges of masses can be cross-referenced with **Figure 3**. Species codes are defined in **Table 1**.

mass (+1.19 g) and higher fat mass (+0.20 g) than those species typically breeding further north. There was no evidence of any body mass-by-breeding region interaction. In addition, there was no evidence that migration distance improved model fit for lean mass ($\chi^2 = 0.28$, df = 3, $P = 0.964$) or fat mass ($\chi^2 = 0.33$, df = 3, $P = 0.955$).

Three species, Ruby-crowned Kinglet (*Regulus calendula*, adult = 37, juvenile = 181), Swainson's Thrush (*Catharus ustulatus*, adult = 37, juvenile = 128) and White-throated Sparrow (adults = 51, juvenile = 155), had sufficient autumn data to fit linear mixed models with factors age and body mass, and random effects for species and years (2009 and 2019). In the analysis of lean mass, random intercepts for species were selected over random intercepts and slopes (LRT: $\chi^2 = 0.49$, df = 2, $P = 0.784$). Compared to this model, there was evidence to support the inclusion of age and the age-by-body mass interaction as fixed effects ($\chi^2 = 7.24$, df = 2, $P = 0.027$), and some support to retain the interaction over a model with main effects only ($\chi^2 = 2.89$, df = 1, $P = 0.089$). Overall, juveniles had slightly greater lean mass than adult birds, and the scaling relationship showed slightly greater hypoallometry of lean mass in adults ($\beta = 0.729$) than in juveniles ($\beta = 0.740$; difference in $\beta = 0.011$ [CI: −0.002, 0.024]; **Figure 6**). For a 20% increase in body mass, this corresponded to a predicted increase in lean mass of 14.2% in adults and 14.4% in juveniles. In the analysis of fat mass, random intercepts for species were selected over random intercepts and slopes (LRT: $\chi^2 = 0.89$, df = 2, $P = 0.640$). There was no evidence to support the inclusion of age class ($\chi^2 = 0.23$, df = 1, $P = 0.636$).

TABLE 4 | Random effects in scaling relationships with body mass for songbirds stopping over at Long Point, Ontario.

Scaling model	Random component	SD
Lean mass vs. body mass		
Species (*n* = 25)	Intercept	0.171
	$\log_{10}$(body mass)	0.106
Year:Season (*n* = 9)	Intercept	0.009
Residual		0.019
Fat mass vs. body mass		
Species (*n* = 25)	Intercept	1.537
	$\log_{10}$(body mass)	1.008
Year:season (*n* = 9)	Intercept	0.096
Residual		0.198

For species, random intercepts and slopes are shown.

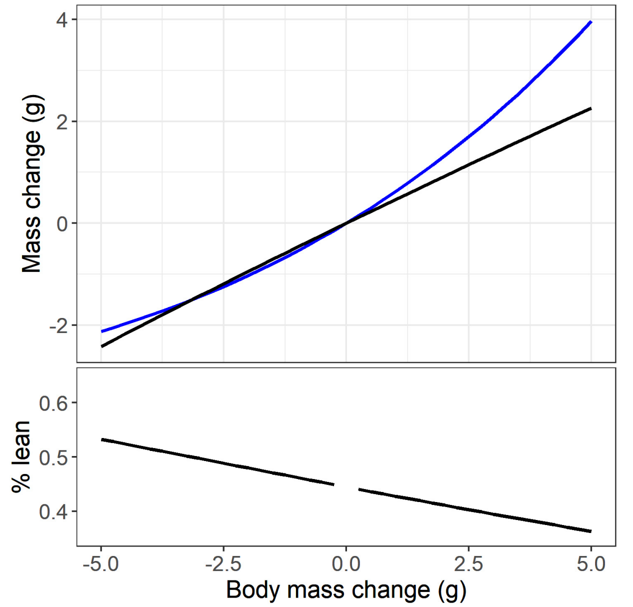

FIGURE 5 | The interaction between the predicted hyperallometry of fat mass change (blue line) and the predicted hypoallometry of lean mass change (black line) relative to body mass change for a White-throated Sparrow of median body mass. The % change of total mass that is lean mass is shown in the bottom panel. The observed range of body mass for White-throated Sparrow was 12.3 g.

Body Composition Dynamics of Recaptured Individuals

In recaptured individuals mass change varied from −5.1 to 4.6 g, with 9 (6%) birds having 0 mass change and 60 (41%) having positive mass change. During data exploration, an additional 3 influential values for percent fat or lean mass change were removed. In contrast to the expectation of linear relationships, which follows from the allometric relationships observed using cross-sectional data, the relationships between percent change in body mass components and percent change in mass were non-linear, and conformed to quadratic relationships [fat: $F_{(2,139)} = 87.9, p < 0.0001, R^2 = 0.56$; lean: $F_{(2,139)} = 112.2, P < 0.001, R^2 = 0.62$]. Thus, percent change in fat mass showed greater hyperallometry than seen for single scan analysis, with greater fat gain than expected for birds that gained a lot of mass. On the other hand, percent change in lean mass showed greater hypoallometry than expected from the single scan analysis, with less lean mass gain than expected for birds that gained a lot of mass (**Figure 7**). There was little evidence for the inclusion of season or species grouping (White-throated Sparrow vs. others) in these models (all $P > 0.165$).

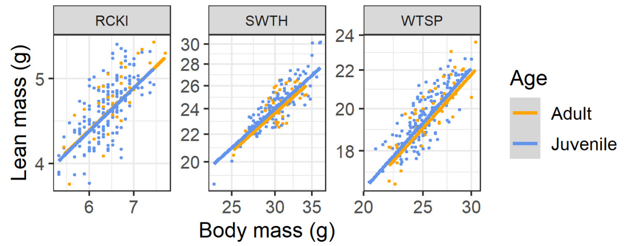

FIGURE 6 | Scaling of lean mass with body mass for 3 species of songbirds stopping over at Long Point, Ontario, highlighting differences between age classes in autumn. The lines show the linear mixed model predictions for juvenile (blue) and adult (orange) birds when year = 2009. Species codes are defined in **Table 1**.

Predictive Equations

Using data for first captures and subsequent recaptures, median percent fat ranged from 3.2 to 15% among species. Predictive equations for fat (g) are shown in **Supplementary Table 2**. In a few species, collinearity resulted in wing chord or fat score being removed from the model. Among species, R^2 values for these predictive equations ranged from 0.431 to 0.934. When using one-half of the dataset as a training dataset to predict fat values in the remaining data, predicted R^2-values dropped to 0.068–0.844. In part, this reduction in fit could be due to small sample sizes for some species or variation attributable to year and season effects. The higher the R^2 and predicted R^2 values, the more generalizable the predictive equations will be to new data at the same site. These species are indicated in **Supplementary Table 2**.

DISCUSSION

Scaling analysis is an effective method to understand patterns of fat and lean mass deposition and loss using either cross-sectional or longitudinal data. We found that lean mass deposition is significant during stopover in songbirds, and of the magnitude predicted from the losses of lean mass in flight. We also found that the relative gains of fat increase as birds deposit fuel and become heavier, that migration distance and breeding region had minimal effects, and that there were effects of season and age on body composition dynamics. Non-invasive analysis of body composition, in this case by a portable QMR instrument, makes it possible to take measurements of large numbers of birds without harming them. This creates opportunities for novel studies and measurements of rare or at-risk species. Most importantly, QMR analysis allows for longitudinal studies of individuals as they

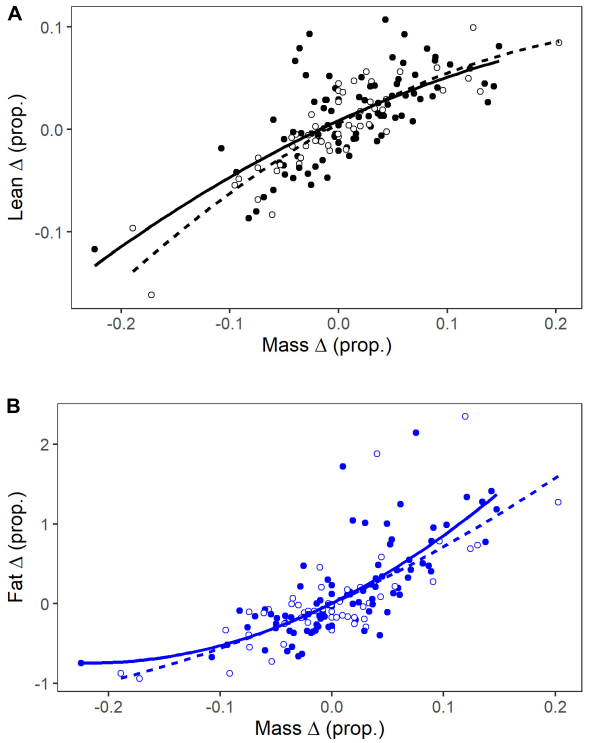

FIGURE 7 | Proportional change in lean mass **(A)** and fat mass **(B)** in relation to proportional change in total mass for recaptured songbirds at Long Point, Ontario. Shown are the quadratic relationships for all birds omitting White-throated Sparrows (solid symbols and lines) and White-throated Sparrows alone (open symbols and dashed lines).

change body mass and composition, as well as cross-sectional studies at the population level.

Differential Allometry of Lean Mass and Fat

Body structural size, as indicated by wing chord, strongly affected total body mass and lean mass, but did not explain much variation in fat. This result is expected because lean mass makes up the majority of body mass, and because muscle masses and the sizes of organs that support them must increase with overall skeletal structure, while fat is an energy storage tissue that is quite dynamic and only weakly determined by structural size. Body mass was a stronger predictor of both lean and fat mass than was wing chord, as evidenced by the much larger proportion of variation explained by fixed effects than random effects in models where body mass was the independent variable. This indicates that structural morphology does not explain lean and fat mass as much as the total mass of tissue that a bird has. Random effects were always important, with species being much more influential than year-season. This shows that species varied in body components and how they scaled with size and mass, whereas variation due to sampling episode was less important.

Among individuals, lean and fat mass showed fundamentally different patterns of allometric scaling with body mass. The general pattern across species was that fat mass increased hyperallometrically with body mass, increasing by about 140% for every 20% increase in body mass. Lean mass, in contrast, showed hypoallometry and increased by only 12–13% for every 20% increase in mass. Within recaptured individuals, where body structural size is constant, the patterns of change in lean and fat mass resembled the population-level analysis, but showed greater hyperallometry of fat and greater hypoallometry of lean mass. This finding is reminiscent of the pattern described previously where population-level regression analyses may overestimate the deposition of lean mass and underestimate fat deposition due to the fact that some of the variation in lean mass is related to structural size (Lindström and Piersma, 1993; van der Meer and Piersma, 1994). However, on the whole, the individual level analysis supports the population-level results, and taken together they demonstrate that lean mass deposition is common and substantial (30–50% of mass change; **Figure 5**) when songbirds refuel at stopover. Lindström and Piersma (1993) estimated that lean mass deposition ranged from 2 to 60% of mass gain across a variety of shorebirds and waterfowl. Other studies report lean mass deposition of similar magnitude (Karasov and Pinshow, 1998; Guglielmo and Williams, 2003; Guglielmo et al., 2011; Seewagen and Guglielmo, 2011; Wojciechowski et al., 2014). Thus, our findings are congruent with previous observations and with first principles based on the rate of catabolism of lean mass in flight.

The intraspecific allometric scaling of lean mass and fat with body mass means that changes are multiplicative, and the amount of each body component that is deposited or lost with changing mass depends on the starting body mass. The hypoallometric scaling of lean mass with body mass indicates that increases in lean mass with increasing total mass will be steeper at low body masses and tend to plateau toward large body masses, similar to a breakpoint pattern. The strong hyperallometry of fat shows that small amounts of fat are deposited when body mass is low and fat deposition accelerates at high body masses, again producing a breakpoint-like pattern. These patterns may relate to birds needing to rebuild the gut and liver before rapid fat deposition can occur (Karasov and Pinshow, 2000; McWilliams and Karasov, 2001; Bauchinger et al., 2005), or to the functional benefits of additional muscles and organs plateauing while fat stores continue to increase to extend flight distance. If we had birds that reached extreme fat levels, such as at major ecological barriers, we may have in fact detected a plateau in lean mass at very high body masses (Carpenter et al., 1993; Wojciechowski et al., 2014), but songbirds refueling at Long Point generally carry modest fat loads.

Using recaptured birds measured longitudinally through time eliminates the confounding variation of body structural size, and so allows one to examine directly changes in lean mass and fat as individual birds refuel at stopover. However, recaptures are rare—145 of 3,549 birds in our study—and they may represent a biased sample of birds that remain at the stopover longer than randomly sampled individuals. Thus, recaptures could be low-quality birds or individuals that were stressed by initial capture. In fact, about half of our recaptured birds lost mass between captures, meaning that many in the sample had not been able to refuel. This provided an opportunity for us to fully model mass gain and loss, and we found very similar results as the analysis of the larger population-level, single-capture dataset. Using single captures has the added benefits of larger sample sizes of more species, and it can be used to test for effects of important covariates, such as season, age, and migration distance.

Season, Age, and Migration Distance

Seasonal differences in body composition and its dynamics were subtle but likely to be biologically important. Previous studies have reported larger fat loads in songbirds during spring migration (Seewagen and Slayton, 2008; Schmaljohann et al., 2018), including at Long Point (Kennedy, 2012). In our study, at the same body mass, spring birds were fatter and showed greater hyperallometry of fat than migrants in the autumn. Greater fat loads in spring may be adaptive by providing birds more energy to cope with colder and more variable weather during migration, which can be a factor limiting migration timing and success (Drake et al., 2014), or as insurance against unpredictable food supply at the breeding destination (Sandberg and Moore, 1996; Drent, 2006). An analysis of 48 species over 17 years showed that refueling conditions at Long Point are poorer in the spring than in autumn (Dunn, 2001). Spring migrants under time constraints to reach the breeding areas also may prioritize fat storage to support longer flights (Dierschke et al., 2005), or may be following a general policy whereby they depart stopover sites with greater fuel loads (Hedenström and Alerstam, 1997). While the autumn samples were often dominated by juveniles, it appears that the seasonal differences were robust as they were larger than the age differences we detected and were apparent when only adults were compared between seasons in the White-throated Sparrow. The greater relative lean mass deposition of autumn migrants suggests that they may have greater dietary protein requirements than spring migrants, despite the former's tendency toward greater frugivory (Parrish, 1997). Thus, invertebrates and other high protein foods are likely to be important for refueling migrant songbirds in both spring and autumn. Focused physiological studies are needed to explore whether there are seasonal differences in the metabolism and routing of nutrients to fat and lean mass.

Although the magnitude of the effect was small, juvenile Swainson's Thrushes and White-throated Sparrows tended to have greater lean mass and to deposit relatively more lean mass than adults. There was no age effect in the much smaller Ruby-crowned Kinglet. This finding supports the results of McCabe and Guglielmo (2019) who found using salvaged carcasses that juvenile songbirds of three of four species studied (including Swainson's Thrush and White-throated Sparrow) had 8–9% greater lean mass than adults. More noticeable was that in all four species studied, juveniles allocated more of their lean mass to digestive organs than adults, and enlarged digestive systems have been reported in juveniles of other birds (Hume and Biebach, 1996; Guglielmo and Williams, 2003). The gut and associated organs like the liver have much greater metabolic turnover than other lean tissues (e.g., muscle; Bauchinger and McWilliams, 2009), and young animals that are still growing and maturing generally have greater rates of protein turnover than adults (Yousef and Johnson, 1970). Juvenile White-throated Sparrows and Swainson's Thrushes had higher basal metabolic rates than adults, which may reflect the added metabolic costs of these active tissues (McCabe and Guglielmo, 2019), and they converted less ingested energy into stores. In our study, QMR allowed us to detect a slight difference in total lean mass, but not to measure how lean mass was allocated within birds, which would require magnetic resonance imaging (MRI) or other imaging technologies (ultrasound or micro-CT). If juvenile songbirds have more lean mass that turns over more rapidly, then they may require greater protein intake during refueling than adults. More studies are needed on songbird protein requirements (Langlois and McWilliams, 2010), protein turnover, lean mass flexibility, and its energetic costs in juvenile songbirds.

Predictive Equations

The species-specific predictive equations for fat mass (fat mass $\sim$ body mass + wing chord + fat score) can be applied when QMR data is not available, but the accuracy of these predictions will depend on several factors. Accuracy will be maximized when using predictive equations with high R^2-values, and when the covariance structure among wing chord, body mass, and fat score are the same (Dormann et al., 2013). Thus, the predictive equations will be most applicable when applied to the same site as we used. For example, it should now be possible to evaluate annual trends in predicted fat using long-term migration monitoring datasets from Long Point Bird Observatory or nearby stations. When applying these predictive equations in a different context, care should be taken to evaluate the covariance structure of the variables and to ensure variables (e.g., fat score) are measured in a consistent way. Ideally, QMR could be used to develop predictive equations at other banding sites based on training datasets.

CONCLUDING REMARKS

Scaling analysis has been a standard method used by comparative physiologists and morphologists for many decades to describe how body components and other traits vary with body size (Schmidt-Nielsen, 1984). Many previous studies of fuel deposition in migratory birds have used various regression methods on raw fat and lean mass data, yet we should expect body composition changes to be non-linear. Our study shows how the allometric scaling of lean and fat mass can be analyzed in a straightforward manner using $\log_{10}$ transformation and linear mixed-effects models to allow for random effects and test important covariates of interest. The allometric slopes (ß) are very intuitive to understand and nicely describe the multiplicative nature of changes in lean mass and fat as total body mass changes. Regarding breakpoints that may exists at the extremes of body mass, these could still be explored in scaling analysis. But for migrant songbirds refueling at a typical stopover, and not facing or arriving after a major ecological barrier, standard allometric scaling analysis will adequately describe the dynamics of fat and lean mass. When combined with non-invasive technologies to measure body composition, such as QMR, this approach should facilitate a wide variety of new studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Western Ontario Animal Care Committee.

AUTHOR CONTRIBUTIONS

CG co-designed all of the studies which provided data, co-wrote the manuscript, and assisted with data analysis. YM co-designed several of the studies which provided data, reanalyzed all the data, and co-wrote the manuscript. LK helped design the initial study in 2009, analyzed the data, contributed sections to the manuscript, and edited the manuscript. JD, JB, and AB contributed data to the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.787623/full#supplementary-material

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Changes in Behaviour and Proxies of Physiology Suggest Individual Variation in the Building of Migratory Phenotypes in Preparation for Long-Distance Flights

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Long-distance migration in birds is a complex syndrome that involves high energy costs and, in some species, substantial physiological re-organisation. Such flexible migratory phenotypes are commonly associated with bird species flying non-stop across vast ecological barriers, where there are few opportunities to stop and refuel *en route*. Prior to making migratory flights, some species have been found to atrophy organs that are not required (e.g., digestive organs) and grow those associated with powering flight (pectora muscles and heart), presumably to optimise costs. However, most studies of this flexibility have required sacrificing study animals and this has limited our capacity to measure individual variation and its potential consequences. Here we investigate the behavioural and, indirectly, physiological adaptation of an arctic breeding long-distance migrant the light-bellied brent goose *Branta bernicla hrota*, during spring staging in southwest Iceland. We use a sequential sampling approach to record behavioural observations and conduct stable isotope analysis of faecal samples from uniquely marked individuals to assess protein catabolism. Individuals showed a three-phase fuel deposition process, with initial slow intake rates followed by hyperphagia and then a period of inactivity immediately prior to migratory departure (despite multiple days with favourable wind conditions). The C:N ratio and δ^{15}N values in faeces were significantly linked to fat deposition during the latter stages and suggests catabolism (reorganisation of proteins) occurring prior to departure. Our results suggest a strategic delay in migratory departure to enable reorganisation into a flying phenotype and that the extent of this varies among individuals.

Keywords: migratory birds, spring staging, phenotypic flexibility, atrophy, behaviour

INTRODUCTION

Long-distance migration is a remarkable behaviour involving the cyclical movement of animals often directed by seasonal variability of global resources (Piersma et al., 1999; Saino et al., 2010; Soriano-Redondo et al., 2020). Despite the high energetic costs of travelling such long distances, it is a strategy that enables species to expand their range, exploiting specific habitat niches best suited to their current life history stage (Hedenström, 2008). Although widespread in the animal kingdom, it is commonly associated with bird species and often characterised by extensive non-stop flight over vast ecological barriers (Saino et al., 2010; Hou and Welch, 2016). This can be physiologically demanding as sparse opportunities for groundings cause extended periods of fasting while still maintaining high metabolic activity (Lindström and Piersma, 1993; Piersma and Gill, 1998; Bauchinger and Biebach, 2001; Bauchinger et al., 2005; Scott et al., 2015; Remisiewicz et al., 2016).

To deal with the high energetic costs of migration, birds often rely on staging sites along their migration route. These act as stepping stones where individuals can rest and refuel before resuming the journey (Mehlman et al., 2005). Staging and refuelling birds undergo significant physiological changes, switching between wholly catabolic states during flight to a largely anabolic state during stationary periods (Landys-Ciannelli et al., 2003). In an effort to efficiently replenish energy stores and gain substantial amounts of adipose fat, birds will enter a state of hyperphagia upon arrival at the staging site (Lindström, 2003). However, fat accumulation is not the only physiological change occurring during a stopover (Karasov and Pinshow, 1998; Battley et al., 2000; Gannes, 2002). The avian digestive system is known to be flexible with associated muscles and organs undergoing hypertrophy during hyperphagic feeding and atrophy during flight induced fasting (McWilliams and Karasov, 2001; McWilliams et al., 2004). Likewise, organs involved in maintaining high performance sustained migratory flights such as pectoral muscle and heart, undergo hypertrophy in the days prior to departure from the staging grounds (Marsh, 1984; Gaunt et al., 1990; Evans et al., 1992; Piersma, 1998; Piersma et al., 1999; Lindström et al., 2000; Bauchinger and Biebach, 2005; Dietz et al., 2007). Due to the excessive metabolic cost needed to maintain the digestive system and the energetic cost associated with carrying its weight, Piersma (1998) suggests that long-distance migrants with no immediate necessity for these organs gain an advantage in reducing them before embarking on a migratory flight. For example, bar-tailed godwits (*Limosa lapponica*), increase stomach mass by 30% during the first half of spring staging only to decrease its mass by 20% before migratory departure (Piersma and Lindström, 1997; Piersma and Gill, 1998). Jehl (1997) provides further evidence for this migratory strategy of phenotypic flexibility, finding eared grebes (*Podiceps nigricollis*) pre-departure reduce their stomach, liver and intestine by up to 57%.

The energetic constraints for avian migrants vary according to environmental conditions encountered *en route* and their life history strategies, the latter being particularly acute for large-bodied High Arctic breeders. Given the scarce foraging opportunities available on arrival at the breeding site, they are often capital breeders, relying on endogenous resources to initiate and finance breeding (Drent et al., 2007; Drent and Daan, 1980; Jaatinen et al., 2016). While Arctic migrants can benefit from 24-h daylight and abundant food resources over summer, this is restricted to a narrow period where conditions are optimal to successfully breed and fledge offspring (Poussart et al., 2000; Drent et al., 2007; Jónsson et al., 2009). Reproductive success is shown to significantly decline with a later laying date, however, eggs laid earlier in the season were found to be formed using a higher proportion of endogenous resources (Lepage et al., 2000; Bêty et al., 2004; Schmutz et al., 2006). This highlights the importance for High Arctic migrants to gain adequate resources at staging sites along the flyway, enabling a timely arrival and rapid initiation of breeding (Hupp et al., 2006; Schmutz et al., 2006).

A number of studies have highlighted the extent of phenotypic flexibility during spring migration in birds [e.g., *Red knot* (*Calidris canutus rogersi*): Battley and Piersma, 1997, (*canutus/islandica*) Piersma et al., 1993; *Great knot* (*Calidris tenuirostris*): Battley et al., 2000, *Eared Grebe*: Jehl, 1997, *Bar-tailed Godwit:* Piersma and Gill, 1998, *Dark-eyed junco* (*Junco hyemalis*): Ramenofsky et al., 1999, *Garden warbler* (*Sylvia borin*): Biebach, 1998; Bauchinger et al., 2005, *Greater snow goose* (*Anser caerulescens atlanticus*): Gauthier et al., 1984]. However, our understanding of this phenomenon has often come from a small number of sacrificed individuals, as shown in the above-mentioned studies, or from controlled feeding and simulated migration experiments (e.g., Karasov et al., 2004; Pierce and McWilliams, 2004). Research specifically interested in flight physiology, measuring changes in protein and lipid catabolism are able to use non-invasive sampling techniques *in situ* (e.g., *pigeons* (*Columba livia*)*:* Hatch et al., 2002, *Western sandpiper* (*Calidris mauri*): Guglielmo and Williams, 2003]. However, many of these studies remain cross sectional, only measuring an individual's physiological state once. These methodologies inhibit the study of within-individual level variation and the potential link to downstream fitness consequences (Piersma and Gill, 1998). Our study presents a route to overcoming difficulties in longitudinal sampling by using behavioural observations and stable isotope analyses of faecal deposits as proxies for large scale reorganisation of internal tissues. We sequentially sampled from marked light-bellied brent geese (*Branta bernicla hrota*) during their spring migration staging in southwest Iceland. While stable isotope measures: C:N ratio, $^{13}C/^{12}C$ ($\delta^{13}C$) and $^{15}N/^{14}N$ ($\delta^{15}N$) have been extensively used to characterise the diets of animals, they can also be used to infer physiological reorganisation (Hobson and Clark, 1992; Inger and Bearhop, 2008). Birds experiencing nutritional stress regardless of diet are known to mobilise and deposit proteins between bodily tissues resulting in additional isotopic discriminations and the enrichment of heavier nitrogen isotope in faeces (Hobson and Clark, 1992). By considering stable isotope analyses alongside food intake rate there is the potential to infer rates of protein catabolism in staging migratory birds. This in turn allows us to assess the extent to which individual animals exhibit phenotypic flexibility, through the reorganisation of body proteins in preparation for a period of migratory flight and subsequent fasting. The east Canadian High Arctic population of Light-bellied brent geese are an excellent model system for such a study. They are herbivorous capital breeding migrants, staging in west Iceland prior to a long-distance flight into the breeding grounds and there are a large number of individually marked birds in the population making it easy to follow individuals through time (Inger et al., 2010).

We predict that phenotypic flexibility will be evident in the light-bellied brent geese during their one staging event on spring migration, manifesting as (1) elevated foraging and locomotor activity soon after arrival, (2) increased δ^{15}N and decreased C:N ratio in feaces just prior to migratory departure, and (3) a period of reduced foraging and locomotor activity prior to departure irrespective of favourable wind conditions being present.

MATERIALS AND METHODS

Study System

We conducted our study in Álftanes (southwest Iceland, 64° 06′N, 22° 00′W), a main spring staging site for light-bellied brent geese of the East Canadian High Arctic population (ECHA). We carried this study out during the brent goose spring migration staging (01/05–27/05/2017), a critical period of intense foraging, where birds accumulate energy stores for migratory flight and breeding (Inger et al., 2008, 2010). In late April, the population has a staggered migration from wintering grounds along the Irish coasts to staging sites in west Iceland (Inger et al., 2008). They typically resume migration *en masse* approximately one month later to their breeding grounds in the Canadian High Arctic (Gudmundsson et al., 1995; Clausen et al., 2003; Robinson et al., 2004). The ECHA population amounts to approximately 35000 individuals of which over 10% are uniquely marked with coloured *Darvic* leg rings displaying individual alphanumeric combinations (Cleasby et al., 2017). We conducted this study on marked individuals only and all observations in this study were made from inside of a vehicle, through an Opticron HR 80 GA ED telescope with an SDLv2 40936 zoom eyepiece (×54 zoom) and Meopta Meo Star B1 12 × 50 HD binoculars, causing minimal disturbance to geese. We observed birds at opportunistically selected field sites around Álftanes from 6.00 h to 22.00 h.

The age of geese (juvenile/adult) was visually determined in the field by observation of plumage colouring along the edges of wing coverts, clearly distinguishing juveniles from adults (Reed, 1993). We identified relationships between individuals [partners/family with parent(s) and offspring/single adults/single juveniles] by observing proximity and cohesion during birds' movements, which was found to represent a reliable method of identifying first-order relatedness among individuals (Harrison et al., 2010). We recorded flock size and considered individuals at least 20 goose lengths apart as a different flock.

Abdominal Profile Index

We opportunistically re-sighted marked birds and recorded fat deposition on foraging geese by visually determining abdominal profile indices (API; Owen, 1981; Boyd et al., 1998; Clausen et al., 2003; Madsen and Klaassen, 2006) on a seven-point scale (1–7; 1 being lowest and 7 the highest score; Inger et al., 2008) with accuracy of 0.5. The API score is linearly related to body mass and highly correlated with the size of abdominal fat reserves (Fox et al., 1999; Madsen and Klaassen, 2006). It is commonly used on geese and shorebirds as they are known to predominantly store energy in their abdominal cavity (Owen, 1981; Wiersma and Piersma, 1995; Boyd, 2000; Tinkler et al., 2007). We used APIs as an indication of body condition and weight gain. We have evidence from this population and other closely related taxa that it correlates with body mass and subsequent reproductive success (Ebbinge and Spaans, 1995; Tinkler et al., 2007; Inger et al., 2008; Harrison et al., 2011; Harrison et al., 2013).

Peck and Pace Rate

We measured foraging behaviour of individual geese through focal sampling of peck and pace rates. Identifiable geese were initially randomly selected then resampled each week of the staging period. We classified pecks as movement of the beak toward the ground, thereby touching the substrate, and paces as every step taken by an individual (Inger et al., 2006a). We used tally counters to record the number of pecks and paces over 1–2 min and obtained a rate by dividing the number of pecks/paces by the total length of observation. We did not include observations in which birds simultaneously did not peck nor pace in this study. Additionally, we recorded length and occurrence of sitting behaviour when geese sat on the ground during a focal observation.

Stable Isotopes

We used faecal sample collection of marked geese for stable isotope analyses. Given brent geese feed on saltmarsh (marine) and terrestrial grasses, which are isotopically distinct with δ^{15}N values being lower in terrestrial grasses and δ^{13}C values being considerably higher in marine grasses, we classified all field sites accordingly accounting for the resulting difference in carbon and nitrogen stable isotope ratios in faecal samples (Inger et al., 2006b). For collection of faecal samples, we split the field season into three time periods: *early* (May 1–10) reflecting arrival and the onset of hyperphagia, *mid* (May 11–19) reflecting a full hyperphagia and *late* (May 20–27) reflecting the cessation of hyperphagia and preparation for migration. We chose these time periods in response to the feeding rate of geese measured across the spring, i.e., initial increase of feeding rates and API (onset of hyperphagia), reaching a peak toward the end of the staging season (full hyperphagia) and a subsequent drop of feeding rates and API until departure (cessation of hyperphagia). Such a mass trajectory and feeding rate pattern has also been recorded in other *Branta* populations during spring staging, for example in dark-bellied brent geese (*Branta bernicla bernicla*) on their last spring staging site before embarking on a long-distance flight to their Arctic breeding grounds (Dokter et al., 2018). In the *early* period, we collected samples from a range of identifiable individuals and then resampled from these target individuals in each subsequent time period, to track individual variation throughout staging. We collected faecal samples from known individuals with one person observing the targetted goose until it defecated and a second collecting the sample by following directions of the observer through two-way radios. Faecal samples were stored at −20°C in conventional plastic-zip lock bags until analyses.

We subsampled faecal samples along a central horizontal plane of the cylindrical pellet. We freeze-dried samples, ground them into a homogenous powder, and weighed them to 0.7 mg (±0.1 mg) in tin capsules. Stable carbon and nitrogen isotope measurements were determined by a Thermoquest EA1110 elemental analyser interfaced with a Europa Scientific 2020 isotope ratio mass spectrometer (Elemtex Ltd., Cornwall, United Kingdom). Stable isotope ratios were expressed as δ notation, measured in parts per thousand (‰) relative to international standards Pee Dee Belemnite for carbon and air for nitrogen according to the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$ where X is ^{15}N or ^{13}C and R is the corresponding ratio of $^{15}N/^{14}N$ or $^{13}C/^{12}C$. We report stable isotope ratios as $\delta^{13}C$ values for carbon and $\delta^{15}N$ values for nitrogen and data are corrected for sample mass and instrument drift. Measurement precision (standard deviation), based on within-run replicate measures of the laboratory standard, was better than 0.25‰ for $\delta^{15}N$ and 0.21‰ for $\delta^{13}C$ isotopic values.

Wind Conditions

Past studies have shown that long-distance migrants will time their departure to maximise wind support (Grönroos et al., 2012). Using the package "*RNCEP*" (Kemp et al., 2012) in R, we interpolated the 10m above ground U wind (east/west) and V wind (north/south) components (m/s) for a central point on the Álftanes peninsula (64° 06′N, 22° 00′W) using the NCEP/NCAR dataset (Kalnay et al., 1996), provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, United States[1]. Measurements were interpolated on the hour each day between 15 May (15 days prior to departure) and 5 June 2017 (5 days post departure) inclusive.

To model U and V wind as a function of wind support and crosswind we estimated the birds flight direction (heading) on migratory departure using the "*geosphere*" package (Hijmans et al., 2017). By studying the migratory tracks obtained from birds in this population, it is known that light bellied brent geese fly west north west toward Greenland, therefore a reference point on the southeast coast of Greenland was used to calculate heading (65° 42′N, 37° 42′W). Wind support and crosswind were calculated using methods described by Safi et al. (2013) (**Figure 1**). Positive values associated with wind support represented a tail wind and negative values a head wind. Crosswind was taken as an absolute value irrespective of direction (Safi et al., 2013).

Statistical Analyses

We conducted all data analyses using R software version 3.3.1. (R Core Team, 2016). We determined the sex of marked individuals as described in Bodey et al. (2020) by using a discriminant function based on morphological differences between wing length and head-bill of male and female individuals, recorded during catches when darvic rings are attached. All birds that did not fit male or female morphological criteria were classified as "unknown" and were excluded from statistical analyses.

Abdominal Profile Index

To determine the inflection point in observed API values in our dataset, we defined an *nls* function in the native *stats* package using API as a dependent variable and including date in May as an independent variable. For this function, we modelled date in May as sigmoid equation as the sigmoid curve best fit the distribution of API values and therefore allowed for an adequate estimation of the inflection point. We further used a linear plateau model to determine an approximate date during spring staging when weight gain of brent geese attenuated. We defined an *nls* function in the native *stats* package with date and API score as x and y variables along with three parameters aiming to define the best fit intercept, slope, and critical x value (Mangiafico, 2016). Once we determined the critical x value (=date when API started attenuating), we used linear models, fitted with the R package *lme4* (Bates et al., 2014), to test if API and date in May were correlated before API score attenuation and after API attenuation. We used API as a dependent variable and individual sex and date in May as independent variables. We did not add ID of geese as a random effect to the models as each individual only occurred once in the dataset.

Peck and Pace Rate

We used the *glmmTMB* R package (Magnusson et al., 2017) to design global generalised linear mixed models (GLMM) for peck and pace rates of individuals. We fitted both models with negative binomial errors and a log link function. The models had either peck or pace rate as the dependent variable and z transformed: Julian date, API, flock size (log transformed); whether the birds had a partner and/or juveniles, sex, and resource type (marine vs. terrestrial) as independent variables. We used z transformations for API, Julian date and flock size as their scales had a large amount of variation. Furthermore, we included the individual identification (leg ring combination) of each sampled goose as a random intercept term into the models as most geese were sampled multiple times throughout staging. We selected minimal adequate peck and pace rate models (MAMs) by using the *dredge* function of the R package *MuMIn* (Barton, 2009). The *dredge* function returns a list of all possible models with the given predictors, which we set to be ranked by second-order *Akaike information criterion* (AICc) values (Burnham and Anderson, 2004). For both (peck and pace) MAMs we chose the model with the lowest AICc value. To investigate changes in proportional occurrence of sitting behaviour in every week of staging, we used a repeated-measures ANOVA and Tukey's *post hoc* test with *Bonferroni* correction.

Stable Isotopes

To determine the effect of independent variables: API, date, resource type (marine vs. terrestrial) and sex on the dependent variables: C:N ratio, $^{13}C/^{12}C$ ($\delta^{13}C$) or $^{15}N/^{14}N$ ($\delta^{15}N$) values obtained from faecal samples, we fitted linear mixed models using the *lme4* R package (Bates et al., 2014). We added the individual goose ID as a random intercept term in the models. API was included as either the individuals recorded value (1–7) or categorised as below, above or at population average (group mean centring). We determined population average using the

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[1] http://www.esrl.noaa.gov/psd/

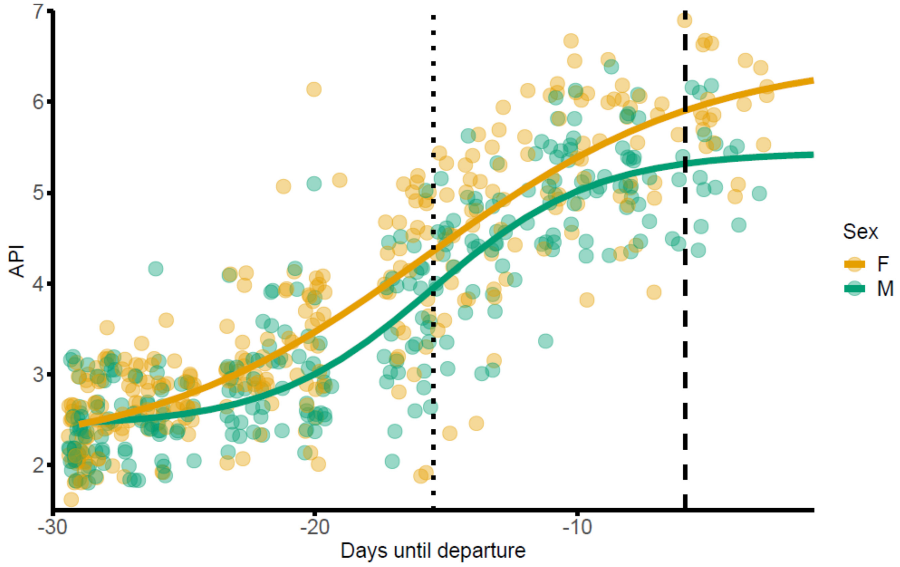

FIGURE 1 | Increase in body condition of brent geese during spring staging in Álftanes, Iceland, as measured by the abdominal profile index (API). Migration to breeding grounds was resumed *en masse* by brent geese on 30/05/17. Females are represented in orange and males in green. The dotted line is the point of inflection, when the rate of weight gain begins to slow (x = 14–15/05/17). The dashed line represents the trough in the second derivative of the sigmoidal function, when the rate of weight gain begins to attenuate (x = 24/05/17).

daily mean API values from re-sighting data collected throughout the staging period in Álftanes. We achieved MAMs by creating all possible models with the given predictors using the R *dredge* function and AICc comparisons. To determine the effects of date, habitat and sex on API (in the Stable isotope dataset), we fitted another linear mixed model with the given responses and individual goose ID as random effect. We used a Tukey's *post hoc* test to determine differences in $\delta^{15}N$, $\delta^{13}C$ and C:N for above/below API individuals across the weeks of spring staging.

RESULTS

We recorded 1,185 re-sightings of 292 uniquely identifiable light-bellied brent geese between May 1–27, 2017 on 9 intertidal saltmarshes and 14 terrestrial field sites. Most birds arrived at study sites before the start of data collection (last days of April) but bird numbers continued to increase during the first week of our study. The bulk of the staging population departed *en masse* from the study area on May 30.

Abdominal Profile Index

The linear plateau model indicated that individuals in this study population reached a plateau in weight gain measured as an abdominal profile index (1–7), of approximately 6 between May 24 and 25 (n = 187 observations, 187 individuals, critical day value = 24.5, 95% CIs: 20.8–28.2, plateau y = 5.7, $p < 0.001$; **Figure 1**). Furthermore, a significant positive linear correlation between API and the number of days into staging was observed until May 24 (n = 172 observations, 172 individuals, $r^2 = 0.70$; est. = 0.15, SE = 0.01, $p < 0.001$) but no such correlation was observed thereafter (n = 13 observations, 13 individuals, $r^2 = 0.41$; est. = 0.05, SE = 0.22, $p = 0.81$). However, after May 24 female geese had higher API compared to males (n = 13 observations, 13 individuals, $r^2 = 0.41$; est. = 22120.96, SE = 0.33, $p = 0.02$). A sigmoid function modelling API by day shows both males and females experience the inflection point in weight gain 15.3 days prior to departure (n = 187 observations, 187 individuals; $r^2 = 0.80$, est. = 15.31, SE = 0.71, $p < 0.001$; **Figure 1**).

Peck and Pace Rate

The top ranking peck rate model, selected by lowest AICc value, included Julian date, the quadratic term of Julian date and resource type as independent variables (AICc = 1757.8, AICc null model = 1759.2; **Table 1**; model selection table in **Supplementary Material 1**). As might be expected given the trajectories, only the quadratic effect of Julian date had a statistically significant negative influence on peck rates during staging (n = 154 observations, 73 individuals, est. = −0.133, SE = 0.054, $p = 0.013$), meaning that peck rates of geese initially increased in the earlier part of the staging period and then decreased again at the end of staging (**Figure 2**).

The best performing pace rate MAM only included Julian date as an independent variable but only differed marginally from the null model (AICc = 1,357.2, AICc null model = 1,357.5; see **Supplementary Material 2** for AICc model selection table). Julian date did not influence pace rates statistically significantly (n = 154 observations, 73 individuals, est. = −0.089, SE = 0.058, $p = 0.121$). Yet, we observed that sitting behaviour differed statistically significantly between the four weeks of staging ($F_{1,3} = 6.727$, $p < 0.001$) and a Tukey *post hoc* test revealed that sitting behaviour was higher in the last week of staging compared

TABLE 1 | GLMM output with peck rate as dependent variable and food resource type, Julian date and quadratic effect of Julian date as independent variables.

	Estimate	Std. error	z value	2.5% CI	97.5% CI	*p* value
Intercept	4.821	0.085	56.690	4.654	4.987	<2e-16
Marine resource type	0.200	0.121	1.660	−0.037	0.437	0.097
Julian date*	−0.110	0.062	−1.760	−0.231	0.012	0.078
Julian date^2*	−0.133	0.054	−2.470	−0.238	−0.027	**0.013**

*The model was fitted with negative binomial error distribution and a log link. *z transformed variables. Bold text reflects statistically significant results.*

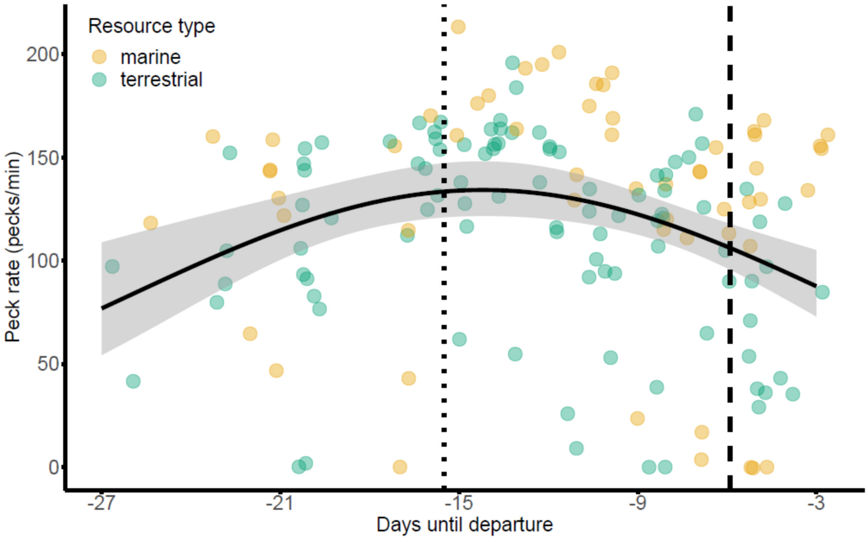

FIGURE 2 | Relationship between peck rates and days until mass departure of light-bellied brent geese from their spring staging site in Álftanes, Iceland. The resource type being fed on during focal observations is coloured as either yellow for marine (saltmarsh) or green for terrestrial (grassland) resources. The black line depicts the quadratic effect of date on peck rates and grey shading represents the standard error. The dotted line is the point of inflection, when the rate of weight gain begins to slow (x = 14–15/05/17). The dashed line represents the trough in the second derivative of the sigmoidal function, when the rate of weight gain begins to attenuate (x = 24/05/17).

to the previous three weeks (week 1 vs. week 4: $p < 0.001$; week 2 vs. week 4: $p = 0.044$; week 3 vs. week 4: p = 0.004).

Stable Isotopes

The best performing MAM for δ^{13}C included habitat type as response variable whereas the best performing MAM for δ^{15}N included habitat type as well as individual API. For C:N the best MAM included API and habitat type as well as their interaction as response variables. All three stable isotope measures showed a significant difference between samples collected on terrestrial or marine habitats, specifically δ^{13}C and δ^{15}N were both higher on marine sites (n = 130 observations, 59 individuals, δ^{13}C: $r^2 = 0.35$; est. = −1.42, SE = 0.17, $p < 0.001$; δ^{15}N: $r^2 = 0.29$; est. = −1.05, SE = 0.36, $p = 0.004$). We found δ^{15}N was statistically significantly higher in birds with higher API (n = 130 observations, 59 individuals, δ^{15}N: $r^2 = 0.29$; est. = 0.33, SE = 0.11, $p = 0.004$, **Figure 3C**) and lower C: N ratios were found in faecal samples of individuals with a higher API (n = 130 observations, 59 individuals, r^2 = 0.28, est. = −1.72, SE = 0.76, $p = 0.02$, **Figure 3A**). However, the results show no significant relationship between an individual's API and habitat type they are feeding on (n = 130 observations, 59 = individuals; r^2 = 0.83, est. = 0.24, SE = 0.14, $p = 0.08$). Using a Tukey *post hoc* test we also found that individuals with higher API than the population average had significantly different δ^{15}N ratios in their faeces compared to those with lower than average API ($p = 0.03$, **Figure 4C**). Both individuals with above and below average API were feeding on similar resources with no significant difference in the δ^{13}C values ($p = 0.99$, **Figure 4B**), there was also no difference in the C: N ratio in faeces ($p = 0.16$, **Figure 4A**).

Wind Conditions

Geese experienced an average wind support of +1.23 m/s between 15 May and 5 June, indicating a tail wind (**Figure 5A**) and a crosswind of 5.21 m/s (**Figure 5B**). On the evening before departure the geese experienced a strong head wind (−6.78 m/s) but this dropped at midnight to −1.11 m/s and became a light tail wind (0.17 m/s) at 06:00 30/05/21. There was an above-average crosswind (6.89–7.51 m/s) present between midnight and 11:00 on the day of departure. Favourable wind support (i.e., tail wind) and crosswind conditions suitable for migratory departure were frequently present between 24 May when API attenuates and 30 May when the geese depart *en masse* and in the days following (**Figure 5**).

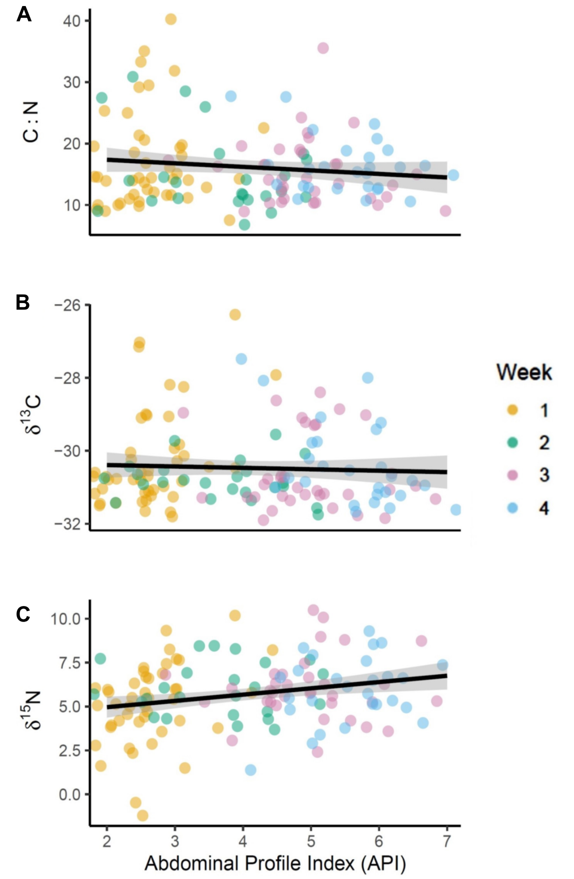

FIGURE 3 | Variation in stable isotope measures **(A)** C:N ratio, **(B)** $\delta^{13}C$, and **(C)** $\delta^{15}N$ associated with weight gain by brent geese during spring staging in Álftanes, Iceland (03/05/2017–27/05/2017). Weight gain measured according to abdominal profile index (API). Data displayed by the week of spring staging when the faecal sample was collected, orange (week 1), green (week 2), purple (week 3), and blue (week 4). Linear regression with standard error (grey shading). An increase in API had a significantly negative effect on C:N ratio ($\chi^2{}_1 = 0.85$, $p = 0.039$, $r^2 = 0.28$) and no significant effect on $\delta^{13}C$ ($p = 0.36$, $r^2 = 0.37$). The $\delta^{15}N$ values were significantly influenced by an interaction between staging week and API ($\chi^2 1 = 8.10$, $p = 0.044$, $r^2 = 0.26$).

DISCUSSION

Light-bellied brent geese demonstrate behavioural and physiological flexibility during their one-month spring staging period in southwest Iceland. The results show that weight gain in brent geese begins attenuating approximately seven days prior to departure from Iceland, with females being on average fatter than their male partners (**Figure 1**). Despite early arrival at breeding grounds being a key driver of hatching success, individuals chose not to capitalise on favourable wind conditions occurring during the last week of staging (**Figure 5**). This suggests that environmental cues are not the only factor driving migratory departure. The observed behavioural changes (**Figure 2**) and stable isotope results across the month of staging (**Figures 3**, **4**) support the hypotheses that brent geese exhibit a period of hyperphagia followed by reduced foraging in response to migratory weight gain requirements. There is also evidence of protein reorganisation occurring prior to departure with a decrease in the C: N ratio (higher proportion of N in the faeces) and higher $\delta^{15}N$ (indicating protein catabolism) toward the end of the staging period. This supports the prediction that physiological adjustments are occurring in preparation for the upcoming flight and capital breeding life history strategy. Of course, there could be other explanations for such a pattern, for example, if resources were abundant and departure date was hardwired due to constraints on the breeding grounds. However, 2017 was a typical year in terms of arrival dates, feeding rates and habitat choice. Moreover, fluctuating conditions on route and at arrival on the breeding grounds would tend to suggest that there should be some ability to optimise departure decisions.

Behaviour

Throughout the month of spring staging in Iceland, light-bellied brent geese significantly changed foraging rates and locomotion behaviour in relation to the time of staging. Initially geese arrived with low API, however despite this, we observed low foraging rates. This is often explained by the "gut limitation hypothesis," whereby an individual experiences slow regeneration of digestive efficiency after a period of fasting (McWilliams and Karasov, 2005). The approximately 1,500 km non-stop migratory flight from Northern Ireland induces a period of fasting likely to cause atrophy of disused organs such as the digestive system. Regeneration of organs requires time and energy, hence an initial slow foraging period upon arrival in Iceland (McWilliams and Karasov, 2005). This has been observed in lesser snow geese, *Chen caerulescens caerulesces* where lower foraging rates were associated with individuals carrying atrophied digestive organs (Ankney and MacInnes, 1978). Furthermore, this hypothesis was also supported in passerine experiments, showing low food intake rates after a period of fasting even when food was offered *ad libitum* (Klaassen and Biebach, 1994; Hume and Biebach, 1996; Klaassen et al., 1997; Karasov and Pinshow, 2000; Karasov and McWilliams, 2005).

We found the time from arrival was the strongest predictor of variation in foraging rates during spring staging. After the initial slow start geese entered a state of hyperphagia, excessively eating in an effort to store enough energy to fuel the continuation of migration and initiation of breeding upon arrival in the High Arctic. As abdominal fat deposits increased so did the foraging rate but both foraging rates and fat gain decreased in the last period of staging, likely due to individuals approaching the target mass required to fuel migration and breeding. Additionally, it becomes more energetically advantageous to maintain a threshold weight as opposed to continue increasing bodyweight (Lindström, 2003), so in this last period before departure fatter individuals were observed sitting more. On average males had a lower threshold weight than females, potentially the result of mate guarding behaviour and a difference in energetic demand experienced by the sexes during long-distance migration and a capital breeding strategy. The reproductive success of female brent geese can change from 8 to 65% with an increase in their body mass at the breeding grounds (Inger et al., 2008). Light-bellied brent geese

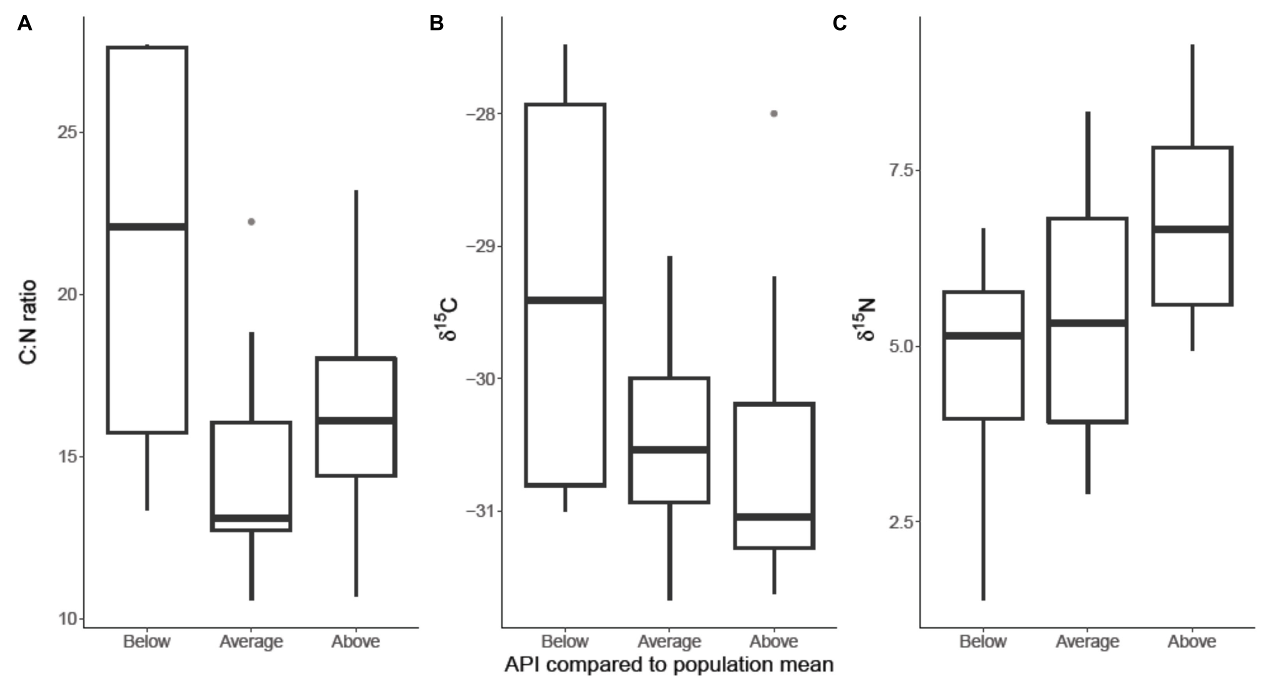

FIGURE 4 | Variation in stable isotope measures **(A)** C:N ratio, **(B)** δ^{13}C, and **(C)** δ^{15}N associated with body condition of brent geese during the final week of spring staging in Álftanes, Iceland (20/05/2017–27/05/2017). Body condition measured as above, below or at population average for abdominal profile index (API). In week 4 individuals with an above-average API excreted significantly higher δ^{15}N values than those with a below-average API (*Tukey post hoc*, $p = 0.025$).

from the adjacent Svalbard/Greenland population are known to lay eggs within a week of arriving (Clausen et al., 2003), highlighting the importance of accumulating large fat deposits prior to departing the spring staging site.

The delay in mass departure despite attenuation of weight gain by both sexes suggests that there are other physiological limitations prolonging the staging period in Iceland. In response to fasting and in-flight starvation waterfowl and wader migrants have been shown to experience protein turnover within their organs (Bauchinger and McWilliams, 2010). Lesser snow geese, a similar-sized waterfowl were found to atrophy organs during a 5–6 day period of decreased foraging rates (Ankney and MacInnes, 1978). This is also commonly seen in migratory wader species such as red knots, *Calidris canutus* found to have a pre-departure turnover of protein, with the digestive organs shrinking and flight muscles (i.e., pectoral) increasing in size (Piersma et al., 1999). This phenotypic flexibility is a potential explanation for the behavioural changes observed in the brent geese during their pre-departure week of spring staging.

Physiology

The stable isotope component of this study in conjunction with the behavioural observations provides further evidence of phenotypic flexibility occurring in the light-bellied brent geese during spring staging in Iceland. We found significant changes in the C:N ratio and δ^{15}N in the faeces of brent geese, in response to reaching peak abdominal fat. Both are indicators of a higher rate of protein turnover that would be expected in animals undergoing fasting and tissue-specific reorganisation, in this instance in preparation for the non-stop migratory flight to their breeding grounds in the Canadian High Arctic.

In comparison to other capital breeding migrants brent geese are physiologically constrained by a large energy requirement to body size ratio (Buchsbaum et al., 1986; Battley and Piersma, 2005). Their solely herbivorous diet has low nutrients and is high in structural components, with cellulose in plant cell walls making it hard to breakdown and digest (Buchsbaum et al., 1986). Therefore, they require a large gizzard and large small intestine to efficiently acquire nutrients and accumulate crucial energy deposits, constituting ∼95% lipids and ∼5% proteins, needed to fuel migration (Sedinger et al., 1989; Jenni and Jenni-Eiermann, 1998; Klasing, 1998; Battley and Piersma, 2005; Rozenfeld and Sheremetyev, 2016). Female geese in particular have a high energetic demand requiring significant fuel accumulation during the month of spring staging to rapidly initiate breeding on arrival and successfully breed. However, a large digestive system takes up crucial space within the abdomen and is metabolically costly during flight and capital breeding. Digestive organs constitute approximately 10% of a bird's lean mass making it a significant burden on wing load (Pierce and McWilliams, 2004). The measured decrease in C:N ratio in brent goose faeces associated with heavier individuals as the staging month progressed is a likely result of a physiological trade-off occurring. As adipose fat is accumulated in the abdomen we can infer that the digestive organs are compromised to maximise available space (Battley and Piersma, 2005). Waterfowl are known to compensate for the low nutrient concentrations in their herbivorous diet by having a long small intestine improving nutrient absorption and

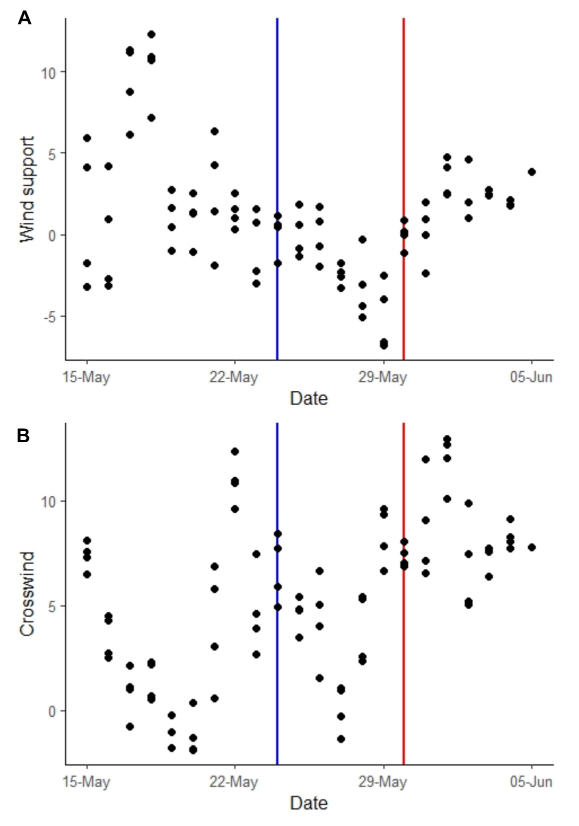

FIGURE 5 | Wind conditions experienced by light-bellied brent geese (LBBG) staging on the Álftanes peninsula between 15/05/17 and 05/06/17. The blue line is the date when LBBG weight gain attenuates (24/05/17) and the red line is the date of their mass departure (30/05/17). Wind conditions were calculated as either **(A)** wind support, with positive values indicating a tailwind and negative values a head wind and **(B)** crosswind was taken as an absolute value irrespective of direction. Calculated with a reference heading direction on the southeast coast of Greenland (65° 42′N, 37° 42′W).

also recycle excretory and endogenous nitrogen in the caecum, improving overall nitrogen balance (Sedinger, 1997; Barboza et al., 2008, p. 156). Given this, a compromised digestive system and resulting reduction in organ size will likely cause a reduction in assimilation efficiency causing the excretion of dietary nitrogen (Barton and Houston, 1993; Sedinger, 1997).

The results also show a significant increase in δ^{15}N of faecal samples as the month progresses and individuals accumulate abdominal fat stores. A higher δ^{15}N is usually indicative of an animal feeding at a higher trophic level (Bearhop et al., 2000; Inger and Bearhop, 2008). However, brent geese are strictly herbivorous and we did not observe a change in their resource use. A study by Fuller et al. (2005) confirmed that δ^{15}N values were not only influenced by dietary intake but also fluctuations in the body's nitrogen homeostasis, suggesting that the increase in δ^{15}N in the goose faeces might have an internal physiological explanation. Migratory birds are known to build, dispense and restore components of their body throughout their annual life history stages, strategically enhancing fuel storage and reducing flight costs (Fox and Kahlert, 2005). Empirical evidence from terminal studies on long-distance migratory water birds demonstrates pre-departure atrophy with up to 50% reduction in digestive tissues occurring at the staging site (Piersma et al., 1993; Jehl, 1997; Piersma and Lindström, 1997; Piersma and Gill, 1998).

The reorganisation of internal organs prior to migratory departure enables a bird to reallocate proteins away from the expensive disused digestive system into the synthesis of flight muscles (McLandress and Raveling, 1981; Jehl et al., 2015). When an animal's body is in a stable feeding state, assimilation of dietary protein tends to result in light ^{14}N being preferentially excreted, resulting in an increase in body tissue δ^{15}N, relative to the diet (Bearhop et al., 2000; Inger and Bearhop, 2008; Krausman and Cain, 2013). However, the enrichment of ^{15}N seen in the faeces of heavier brent geese is commonly found during periods of nutritional stress when higher rates of protein catabolism can often occur (Hobson et al., 1993). In this case, we have inferred that the increase in δ^{15}N represents a controlled reallocation of proteins in preparation for migratory departure.

An alternative explanation for the increase in δ^{15}N in the faeces is that the geese are entering a state of starvation in response to the low food intake, initiating the catabolism of proteins as a source of energy. However, this is unlikely as birds and mammals are known to prioritise lipid utilisation until fat stores drop below a critical limit (Jenni et al., 2000). For migratory bird species, this was shown to be as low as 5–10% of fat remaining and only then do they shift to catabolising protein to meet energy requirements (Cherel et al., 1988; Schwilch et al., 2002). Given that abdominal fat stores remain constant once a threshold weight is reached, it is unlikely that the protein catabolism measured in this study using stable isotope techniques is the result of foraging rates decreasing (i.e., starvation), rather shows phenotypic flexibility in the migratory light-bellied brent geese.

Despite female brent geese carrying a higher energetic burden upon reaching the breeding grounds, the sexes showed no difference between the δ^{15}N values and C:N ratio in their faeces. This highlights the significant cost of carrying potentially redundant organs and the importance of pre-departure protein catabolism and synthesis in an effort to reduce wing load and improve flight efficiency.

CONCLUSION

In this study, we used a non-invasive sequential sampling method to track behavioural and physiological changes in light-bellied brent geese across the month of spring staging. Our results show phenotypic flexibility of this Arctic breeder, in response to the high energetic demands of long-distance migration and a capital breeding life history strategy. A decrease in foraging and locomotion rate in the last week of staging coincided with the attenuation of weight gain. During this last week, we also found fatter individuals had a lower C:N ratio and higher δ^{15}N values in their excretion. All these factors support the prediction that it is beneficial for brent geese to undergo a reorganisation of internal protein, changing from a body prioritising resource acquisition for fat deposition, to one promoting efficient flight. It should be noted that this study was only based on data collected in one spring staging season meaning that it is impossible to make assumptions on the repeatability of the occurrence of the behavioural and stable isotope patterns observed in this study. However, we have no reason to suspect that it was an atypical season. Future directions to improve our understanding of phenotypic flexibility during avian migration should include the non-invasive measurement of gut size throughout the staging period (i.e., using ultrasonography techniques (Vézina et al., 2012). By combining such measurements with behavioural studies and stable isotope data a more complete picture of the phenotypic flexibility during migratory staging could be gained.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the CLES Penryn Ethics Committee.

AUTHOR CONTRIBUTIONS

SB, TH, JS, and XH conceived the project. TH, JS, and SL carried out the fieldwork. TH, JS, and XH analysed the data. TH and JS lead the writing of the manuscript, with contributions from all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Fat Stores and Antioxidant Capacity Affect Stopover Decisions in Three of Four Species of Migratory Passerines With Different Migration Strategies: An Experimental Approach

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During migratory stopovers, birds must make decisions about when and where to travel and these decisions are likely contingent on their fuel stores, food availability, and antioxidant capacity as well as seasonal changes in key environmental factors. We conducted a field experiment on an offshore stopover site (Block Island, Rhode Island, United States: 41°130N, 71°330W) during autumn migration to test the hypothesis that birds with greater fuel stores and non-enzymatic antioxidant capacity have shorter stopovers than lean birds with low antioxidant capacity, and to determine the extent to which this depends on migration strategy. We used a 2 × 2 factorial field experiment (two levels each of available food and dietary polyphenols) with four species of songbirds kept in captivity for 3–5 days to produce experimental groups with different fuel stores and antioxidant capacity. We attached digital VHF transmitters to assess stopover duration and departure direction using automated telemetry. Non-enzymatic antioxidant capacity increased during refueling for Red-eyed Vireos (*Vireo olivaceus*) and Blackpoll Warblers (*Setophaga striata*) fed *ad lib* diets, and for *ad lib* fed Hermit Thrushes (*Catharus guttatus*) supplemented with polyphenols, but not for Yellow-rumped Warblers (*Setophaga coronata coronata*). Glutathione peroxidase (GPx) decreased during captivity and was influenced by dietary treatment only in Red-eyed Vireos. Oxidative damage decreased during captivity for all species except Yellow-rumped Warblers. Stopover duration was shorter for Vireos and Blackpolls fed *ad lib* as compared to those fed maintenance. *Ad lib* fed Hermit Thrushes supplemented with polyphenols had shorter stopovers than those fed *ad lib*, as did thrushes fed at maintenance and supplemented with polyphenols compared with those fed at maintenance alone. There was no influence of condition on stopover duration for Yellow-rumped Warblers. Departure direction was not strongly related to condition, and birds primarily reoriented north when departing Block Island. Thus, fat stores and oxidative status interacted to influence the time passerines spent on stopover, and condition-dependent departure decisions were related to a bird's migration strategy. Therefore, seasonal variation in macro- and micro-nutrient resources available for refueling at stopover sites can affect body condition and antioxidant capacity and in turn influence the timing and success of migration.

Keywords: oxidative stress, condition-dependence, fruit, glutathione peroxidase, micronutrients

INTRODUCTION

Almost 19% of all extant bird species (~1,855 species) undergo seasonal migrations, maximizing their fitness by taking advantage of spatially distinct habitats that vary in resources, environmental conditions, predation, parasites, or competition (Steadman, 2005; Somveille et al., 2013, 2015). Although some avian species are capable of traveling thousands of kilometers during a migratory flight (Gill et al., 2009; DeLuca et al., 2019), most migratory journeys, especially among passerines, are characterized by shorter flights punctuated by longer rest and refueling periods on stopover (Pomeroy et al., 2006; Seewagen and Guglielmo, 2010; Chernetsov, 2012; Covino et al., 2015). These stop-and-go migration strategies include alternately building and expending energy and nutrients at stopover and during flight, respectively (Cohen et al., 2012; Guglielmo, 2018; Schmaljohann and Klinner, 2020). The rate at which birds rebuild body condition likely influences the time they spend at a given stopover and may depend on available resources, and the migration ecology of a given species (Cohen et al., 2012; Toews et al., 2014; DeLuca et al., 2019; Packmor et al., 2020). Understanding how the behavioral decisions of migrants at stopover sites are influenced by their physiological condition and ecology requires field experiments that directly manipulate condition of individuals for multiple species at a given stopover site.

In general, birds stop during migration to rest and refuel prior to their next migratory flight, although doing so at a high rate may expose an individual to risk of predation (Sapir et al., 2004; Pomeroy et al., 2006; Woodworth et al., 2014) and may depend on the quality of the habitat (Smith and McWilliams, 2010; Oguchi et al., 2017; Clark and Seewagen, 2019). Prior research has highlighted how energetic condition can be a driving force behind departure decisions from stopover sites (Wikelski et al., 2003; Eikenaar et al., 2014; Smith and McWilliams, 2014; Deppe et al., 2015). Additionally, energetic condition and habitat quality can influence the direction a bird departs a stopover site (Deutschlander and Muheim, 2009; Covino and Holberton, 2011; Schmaljohann et al., 2011). For example, migrants with larger fat stores in the Gulf of Maine more regularly departed in a seasonally appropriate direction rather than departing on a temporary reverse migratory flight or a regional-scale movement (Covino and Holberton, 2011). However, the evidence for a relationship among fuel stores, time on a stopover site, and/or direction of subsequent flights is generally derived from an observation of the body condition of an individual migratory bird at capture and the amount of time it spends at that stopover after release (Seewagen and Guglielmo, 2010; Eikenaar et al., 2016b; Schmaljohann and Eikenaar, 2017). We know of only two studies to date that have directly manipulated fuel stores in migratory birds to examine how body condition affects stopover duration (Smith and McWilliams, 2014; Dossman et al., 2018). Hermit Thrush (*Catharus guttatus*) fed *ad libitum (ad lib)* and thus with large fat stores were twice as likely to depart a stopover site than lean birds fed a maintenance ration, and those fed *ad lib* were more likely to depart the stopover site in a seasonally appropriate direction (Smith and McWilliams, 2014). Experimentally food-restricted American Redstarts (*Setophaga ruticilla*) were four times less likely to depart a spring stopover site on a given night than paired controls (Dossman et al., 2018). Given the diversity of passerine species that pass-through a specific stopover site during migration (Baird et al., 1959; Parrish, 1997; Smith and Paton, 2011), additional experiments are needed that examine the impact of fuel stores on stopover behavior across multiple species that differ in migration and feeding strategies.

Stopover sites are likely also important for replenishing dietary antioxidant stores and recovering from oxidative damage caused during previous flights (Jenni-Eiermann et al., 2014; Skrip et al., 2015; Eikenaar et al., 2020). During migratory flights, birds have elevated metabolic rates and rely on stored fats as an efficient source of fuel (McWilliams et al., 2004), which may increase the production of reactive species (RS) and propagation of oxidative damage (Costantini, 2014; Skrip et al., 2015; McWilliams et al., 2020). Birds have evolved a multifaceted antioxidant system of endogenous molecules (non-enzymatic and enzymatic) and dietary-derived vitamins to combat or repair the damage created by RS (Cooper-Mullin and McWilliams, 2016). During fall migration, many passerines primarily find dietary antioxidants in the seasonally abundant fruit they rely on for refueling (Sapir et al., 2004; Catoni et al., 2008; Alan et al., 2013). For example, arrowwood (*Viburnum* spp.) fruit is more rapidly consumed than any other fruits on an island stopover site ca. 20 km off the coast of Rhode Island, United States (Block Island; 41°130N, 71°330W), and it has more fat, total lipophilic antioxidants, anthocyanins (a type of polyphenol) and other phenolics compared with other local fruits (Alan et al., 2013; Bolser et al., 2013). At that the same stopover site, circulating non-enzymatic antioxidant capacity was positively correlated with fat stores in Blackpoll Warblers (*Setophaga striata*) and Red-eyed Vireos (*Vireo olivaceus*, Skrip et al., 2015). In two different habitats in northern Columbia, Tennessee Warblers (*Leiothlypis peregrina*) refueling on insects and fruits during spring migration had higher fuel deposition rates than individuals refueling on insects alone (Bayly et al., 2019) and Garden Warblers (*Sylvia borin*) that were opportunistically resampled over several days at a Mediterranean coastal stopover site during spring migration increased circulating non-enzymatic antioxidant capacity while refueling (Costantini et al., 2007). However, the only study to examine an enzymatic antioxidant (glutathione peroxidase, GPx) during migration found that GPx concentration decreased throughout the day in European Robins (*Erithacus rubecula*) that were resting and refueling on stopover (Jenni-Eiermann et al., 2014), indicating that birds may downregulate endogenously produced antioxidants on stopover while increasing non-enzymatic antioxidant capacity from dietary (fruit-derived) antioxidants.

Birds also seem to recover from oxidative damage incurred during migratory flights while on stopover. Across individuals, oxidative damage was positively correlated with fat stores in Red-eyed Vireos and Blackpoll Warblers at a fall stopover site (Skrip et al., 2015), likely a result of consuming oxidatively vulnerable fats (Skrip and McWilliams, 2016). However, within individual Northern Wheatears (*Oenanthe oenanthe*) in the spring, oxidative damage to polyunsaturated fatty acids decreased over 3 days, indicating that birds can recover from the damage incurred during a previous flight (Eikenaar et al., 2020), although the contribution of dietary antioxidants from fruits to oxidative recovery is still unclear. Thus, the ability of a bird to forage effectively for dietary antioxidants, recover from oxidative damage, and to rebuild fat and antioxidant stores on a stopover site may influence behavioral decisions (e.g., length of stay on a stopover, direction of subsequent migratory flights) that affect overall timing and success of migration (McWilliams et al., 2021), but this has not been thoroughly studied.

Although many studies have focused on how amount of food affects fat stores and stopover behavior, no previous study has focused on how variation in dietary antioxidants could influence stopover behavior. We conducted a field experiment on an offshore stopover site in southern New England during autumn migration that involved manipulating the quantity of food as well as its quality (i.e., amount of antioxidants) offered to four sympatric species of migratory passerines (**Table 1**; Blackpoll Warbler, *Setophaga striata*; Red-eyed Vireo, *Vireo olivaceus*; Hermit Thrush, *Catharus guttatus*; Yellow-rumped Warbler, Myrtle subspecies, *Setophaga coronata coronata*). Individuals of each species were separated into two or four experimental groups and kept in captivity for 3–5 days to manipulate their underlying physiological condition. We then tracked these birds with miniature digital VHF transmitters using automated radio-telemetry stations (Taylor et al., 2017) to determine how condition (i.e., fat stores, oxidative status) affected the duration of stopover on the island and departure direction. We tested the following four hypotheses: (1) circulating antioxidant capacity changes with rest and refueling on stopover and the extent and direction depends on availability of dietary antioxidants and the component of antioxidant capacity measured (e.g., non-enzymatic vs. enzymatic), (2) oxidative damage decreases with rest and refueling on stopover but the extent likely depends on fat stores and availability of dietary antioxidants, (3) individuals that are able to build non-enzymatic antioxidant capacity and/or fuel stores (i.e., in better body condition) on stopover are able to depart more quickly and are less likely to exhibit reverse migration (*sensu* Smith and McWilliams, 2014), (4) species that migrate farther (Blackpoll Warblers and Vireos), or those that are more likely to undertake a long-distance flight (Blackpolls) exhibit a different degree of condition-dependent behavior while on stopover.

MATERIALS AND METHODS

Study Site

Block Island (41°130N, 71°330W) is an offshore stopover site located 15.5 km off the coast of Rhode Island, United States and 22.5 km northeast of Long Island, New York, United States (**Figure 1**). This 283 km^2 island serves as a major stopover site for migratory birds in the fall (Reinert et al., 2002). Once on Block Island, migrating songbirds rest and refuel, typically consume large quantities of fruit while stopping over, and then depending on body condition continue migration to the south, or reorient back toward the mainland of Rhode Island (Able, 1977; Parrish, 1997; Bolser et al., 2013; Smith and McWilliams, 2014). Performing this experiment on an island also allowed us to be certain that we were accurately assessing an individual's departure from the stopover site.

Target Species, Capture, and Blood Sampling

During a series of field experiments in fall 2015 and 2016, we manipulated the quality and quantity of food offered to short-term captive individuals of four passerine species and thus produced birds with different body condition prior to release to continue their fall migration (**Table 1**). We chose these four focal species because they are relatively common on Block Island during fall migration, yet they differ in migration strategy. Yellow-rumped Warblers are relatively short-distance migrants and they winter farther north than any other wood warbler species (Hunt and Flaspohler, 2020). Hermit Thrushes are medium-distance migrants that spend the winter in the southern United States and Central America (Smith and McWilliams, 2014; Dellinger et al., 2020). Red-eyed Vireos are long-distance neotropical migrants that typically follow eastern U.S. mountain ranges south until crossing or routing around the Gulf of Mexico to winter in northern South America (Callo et al., 2013; Deppe et al., 2015; Cimprich et al., 2018). All three of these species are presumably able to stopover many times after they depart Block Island (Callo et al., 2013; Skrip et al., 2015). In contrast, Blackpoll Warblers stage in New England during fall migration prior to a 3–5 day non-stop migratory flight over the western Atlantic ocean before reaching a wintering destination in the Caribbean and South America (DeLuca et al., 2015, 2019; Morris et al., 2016; Smetzer and King, 2020). Hatch year (HY) Red-eyed Vireos and Blackpoll Warblers exhibit prolonged stopovers (> 7 days) at coastal New England stopover sites (including Block Island) and so this fall stopover period could represent a crucial ecological bottleneck for these declining species (Smetzer et al., 2017; Smetzer and King, 2018). Although all four species are primarily insectivorous during the breeding season, they shift to a fruit-rich diet in late summer and into fall (Parrish, 1997; Mudrzynski and Norment, 2013; Woodworth et al., 2014; Marshall et al., 2016); however, only Yellow-rumped Warblers are able to consume and digest fruits from the waxy bayberry

TABLE 1 | Total migration distances and experimental treatment groups for Blackpoll Warblers, Red-eyed Vireos, Hermit Thrushes, and Yellow-rumped Warblers.

Species	Breeding area	Wintering area	Total fall migration distance	References	Manipulation treatment
Neotropical migrants					
Blackpoll Warbler (*Setophaga striata*)	Boreal forest (western Alaska to Newfoundland, Nova Scotia and Northern New England)	Northern South America east of the Andes Mountains	3,820–21,600 km (up to 2,770 km non-stop: New England to South America)[a]	DeLuca et al., 2015, 2019	*Ad Lib* Maintenance
Red-eyed Vireo (*Vireo olivaceus*)	Eastern North America	Northern South America (Columbia, Venezuela, Guyana, Suriname, Ecuador, Peru, Western Brazil)	5,264 km (non-stop over Gulf of Mexico)[a]	Callo et al., 2013	*Ad Lib* Maintenance
North and Central American migrants					
Hermit Thrush (*Catharus guttatus*)	North America (above ∼37° Latitude)	North and Central America	0–2,887 km[a,b]	Smith and McWilliams, 2010; Alvarado et al., 2014	*Ad Lib* *Ad Lib* + Dietary Antioxidants Maintenance Maintenance + dietary antioxidants
Yellow-rumped Warbler (Myrtle subspecies, *Setophaga coronata coronata*)	Coniferous forests of Canada and north east United States	Primarily southern United States, Baja, Mexico, and Caribbean	0–∼5,000 km[b,c]	Hunt and Flaspohler, 2020	*Ad Lib* *Ad Lib* + Dietary Antioxidants Maintenance Maintenance + dietary antioxidants

[a] *Estimated based on geolocator studies (cited).*
[b] *Occasionally winters on Block Island, Rhode Island.*
[c] *Coarse estimate based on species range.*

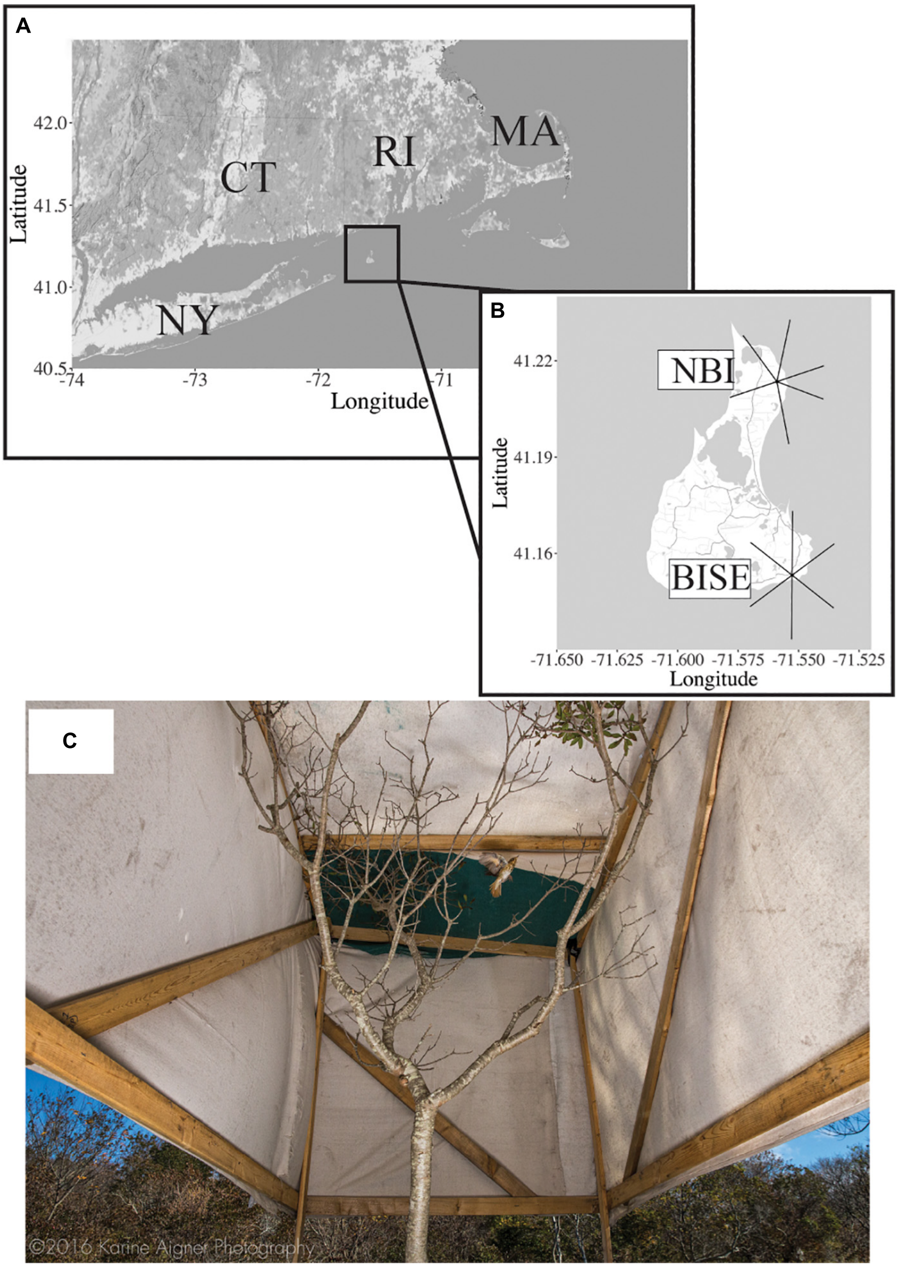

FIGURE 1 | (A) Location of Block Island (41°130N, 71°330W), Rhode Island, United States with **(B)** the antennas of the two automated radio telemetry towers marked (NBI: northern Block Island and BISE: southeast Block Island). **(C)** Picture from the ground of a Hermit Thrush in the soft-release aviary that is entirely covered in canvas except for the bottom 1 m which was left open. Note that this was a re-enactment photo taken during the mid-day; in reality, birds were gently placed in this aviary 1.5–2.5 h after sunset and then were allowed to voluntarily depart, usually at dawn the next day (see section "Materials and Methods" for details). Photo by ©KarineAigner.

(*Myrica* spp.) (Place and Stiles, 1992; Afik et al., 1995; Parrish, 1997; McWilliams and Karasov, 2014) which is quite common on Block Island during fall and winter (Smith et al., 2007).

Mist nets were operated daily on Block Island from 60 min before dawn until sunset on all fair-weather days between September 1 and November 11, 2015 and 2016. When a target species was captured, we drew a up to 150 μL blood sample to measure antioxidant status and oxidative damage at capture (see below for details). Mist nets were monitored every 45–60 min and blood was drawn within 10 min of extraction for 75% of the birds and within 20 min of extraction for all birds (mean ± *SE* for all blood samples: 12.15 ± 1.49 min). All samples were centrifuged within 20 min of bleeding for 10 min at 5,000 rpm, and the separated plasma and red blood cells were flash frozen under liquid nitrogen and later stored at −80°C prior to analysis. After blood sampling, we measured subcutaneous fat score on a 0–8 scale (Kaiser, 1993), muscle score on a 0–3 scale (Bairlein et al., 1995), body mass (± 0.1 g), wing chord (± 0.5 mm) and tarsus (± 0.5 mm). However, we were unable to reliably determine sex for HY individuals of these four species captured during fall migration (Pyle, 1997).

Manipulation of Fuel Stores

Across the ca. 2-month fall season, we used cohort groups of 2 or 4 HY birds of the same species that were (a) captured within the same morning (1 h prior to sunrise to 6 h after sunrise), (b) of similar body size and weight for each species, and (c) all had fat score = 0 and muscle score = 0–1 at capture (see **Table 2**). For Yellow-rumped Warblers and Hermit Thrushes we had 4 birds per cohort (1 in each of the 4 treatment groups) and for Blackpolls and Vireos we had 2 birds per cohort (1 in each of 2 treatment groups). This careful selection of individuals that were captured on the same day in the same initial condition and of similar body size allowed us to maximize the chance these birds arrived on the same day on Block Island and allowed us to effectively control for any changes in response of individuals to the treatments across the fall season. This protocol produced up to eight cohorts of 2 or 4 individuals for each species over the fall migration season (**Table 2**).

We randomly assigned without replacement individuals within a cohort to one of the diet treatment groups (two diet groups for Blackpoll Warbler and Red-eyed Vireo, four diet groups for Hermit Thrush and Yellow-rumped Warbler). We manipulated food availability by providing individuals with *ad lib* food (*ad lib* treatment; Blackpoll Warblers: n = 8, Red-eyed Vireos: n = 8, Hermit Thrush: n = 16; Yellow-rumped warblers: n = 16) or restricting food availability to levels that allowed individuals to only maintain but not gain weight (maintenance treatment; Blackpoll Warbler: n = 8, Red-eyed Vireo: n = 8), Hermit Thrush: n = 16, Yellow-rumped warblers: n = 16). *Ad lib* treatment groups were provided with more live wax moth larvae (hereafter, waxworms, *Galleria mellonela*) than could be consumed per day. Maintenance groups were given enough waxworms to maintain an individual's weight at capture as determined by preliminary studies (Hermit Thrush: 10 g per day; Blackpoll Warblers, Red-eyed Vireos, Yellow-rumped Warblers: 5 g per day). For Hermit Thrush and Yellow-rumped Warblers, we randomly selected half the *ad lib* and maintenance-fed birds to be supplemented each day with a dietary antioxidant. Specifically, these supplemented birds were gavaged once a day with 2.5 ccs elderberry powder (25 g/100 g anthocyanin and > 40 g/100 g other polyphenols: Artemis International, Inc., Fort Wayne, IN, United States) dissolved in water. This antioxidant concentration was chosen based on previous experiments with European blackcaps, *Sylvia atricapilla* (Catoni et al., 2008), and was equivalent to the amount of polyphenols wild birds would consume in berries in a day (Schaefer et al., 2008).

We housed birds for 3–6 days in standard stainless-steel cages (36 cm × 43 cm × 60 cm) in an outdoor aviary with protection from the elements (e.g., wind, sun, precipitation), but exposure to natural photoperiod and temperature fluctuations. Each day we monitored food intake, body weight, fat scores, and muscle scores. We aimed to release birds as soon as the *ad lib* treatment group reached a fat score of at least 6 (across all cohorts occurred in a max of 4 days), but had to delay release by 1 day for 3 of 32 cohorts due to inclement weather. On the day of release, we obtained a second blood sample of up to 150 μl to examine changes in antioxidant capacity and damage within individuals during captivity. We scaled the amount of blood drawn to the size of individuals so that the maximum amount of blood drawn (at capture plus before release) for each individual was less than 2% of their body weight which conforms to current guidelines (Fair et al., 2010; Owen, 2011).

Radio-Tracking

On the day of release, we fit all birds with a digital VHF transmitter (Blackpoll Warblers, Red-eyed Vireos and Yellow-rumped Warblers: Avian NanoTag model NTQB-1; Hermit Thrush: Avian NanoTag model NTQB-2) attached with a leg-loop harness adjusted to body size (Naef-Daenzer, 2007). The combined weight of the tag and the harness was between 0.28 and 0.33 g (<3.0% body weight). Tags transmitted a coded burst at 166.380 MHz every 6.2 s with an expected battery life of about 40 days. We released individuals over time in up to eight cohorts with individuals in each cohort released on the same night. To ensure that the release location was standardized across cohorts and species, we constructed a "soft-release aviary" (following Smith and McWilliams, 2014) around a bayberry shrub (*Myrica pensylvanica*) that was 200 m from their experimental aviary and not visible from any dwelling. The soft-release aviary had a wooden frame (2.5 m tall × 1.5 m wide × 1.5 m long) with a heavy canvas covering with the bottom 1 m of the aviary left open to provide birds an exit when they voluntarily chose to do so (**Figure 1**). We placed birds in the soft-release aviary 1.5–2.5 h after sunset by removing them from their experimental aviary, placing them in a cloth bag for transport to the soft-release aviary (about a 10 min walk). We then, quietly and in the dark, removed each individual from the cloth bag and gently placed them on a branch in the aviary where they remained as we left the area. We visited the soft-release aviary within 30 min of sunrise and, therefore, we know that all birds left the aviary either during the night or within 30 min of sunrise next morning. Based on detections from the antenna facing the soft-release aviary compared to the other antennas on our local automated-telemetry tower (see below), we know that most birds exited the soft-release aviary during or within 30 min after sunrise. Only four of sixteen Blackpoll Warblers and two of sixteen Red-eyed Vireos departed the aviary during the night.

We used automated telemetry receiving stations in the Motus Wildlife Tracking System (Taylor et al., 2017) to determine how long individuals spent on stopover after release and their departure orientation. Importantly, two automated telemetry receivers were active on Block Island during Fall 2015 and Fall 2016 (**Figure 1**): we built a station near the north end of the island (NBI) and the U.S. Fish and Wildlife Service operated a station near the south end of the island (BISE). Both receiver stations consisted of six 9-element Yagi antennas mounted 12 m above the ground. All antennas monitored a frequency of

TABLE 2 | Target parameters used to select which individuals at capture could be included in each cohort per species and the measured values (mean ± *SD*) of these parameters, number of individuals included and the date of capture for each cohort.

Blackpoll Warbler cohorts

	Measurement target:	Wing chord (mm): 71.5	Tarsus (mm): 19	Weight (g): 14.5
Cohort #	**Date captured (*n* in cohort)**	**Measured mean ± *SD***	**Measured mean ± *SD***	**Measured mean ± *SD***
1	9/9/16 (*n* = 2)	71.5 ± 0.50	19.5 ± 0.25	13.2 ± 1.20
2	9/21/16 (*n* = 2)	69 ± 0.50	18.2 ± 1.06	13.0 ± 0.45
3	9/25/16 (*n* = 2)	69.5 ± 1.50	19.2 ± 1.20	13.8 ± 1.30
4	10/3/16 (*n* = 2)	73 ± 1.40	19.1 ± 0.14	14.0 ± 1.20
5	10/3/16 (*n* = 2)	71.2 ± 0.35	18.7 ± 0.57	13.0 ± 1.22
6	10/4/16 (*n* = 2)	71.2 ± 0.35	19.4 ± 0.64	13.4 ± 1.30
7	10/5/16 (*n* = 2)	71.5 ± 0.41	18.4 ± 1.48	14.5 ± 1.25
8	10/11/16 (*n* = 2)	71.0 ± 0.00	19.0 ± 0.25	15.6 ± 1.32

Red-eyed Vireo cohorts

	Measurement targets:	Wing chord (mm): 77	Tarsus (mm): 18	Weight (g): 16.5
Cohort #	**Date captured (*n* in cohort)**	**Measured Mean ± *SD***	**Measured Mean ± *SD***	**Measured Mean ± *SD***
1	9/20/16 (*n* = 2)	78 ± 0.00	18.8 ± 0.28	17.5 ± 0.14
2	9/21/16 (*n* = 2)	77 ± 0.00	17.2 ± 0.07	15.0 ± 0.07
3	9/23/16 (*n* = 2)	76.2 ± 1.06	18.6 ± 0.91	16.5 ± 0.00
4	9/23/16 (*n* = 2)	77.5 ± 0.71	18.4 ± 0.07	16.9 ± 0.42
5	9/24/16 (*n* = 2)	78.5 ± 1.12	18.4 ± 0.50	17.2 ± 0.50
6	9/26/16 (*n* = 2)	76.5 ± 1.52	17.9 ± 0.00	16.1 ± 0.85
7	10/6/16 (*n* = 2)	76.2 ± 1.06	17.1 ± 0.14	17.2 ± 0.45
8	10/7/16 (*n* = 2)	76.5 ± 1.12	18.6 ± 0.00	17.2 ± 1.63

Hermit Thrush cohorts

	Measurement targets:	Wing chord (mm): 89.5	Tarsus (mm): 29.5	Weight (g): 30.0
Cohort #	**Date captured (n in cohort)**	**Measured mean ± SD**	**Measured mean ± SD**	**Measured mean ± SD**
1	10/21/16 (*n* = 4)	90.5 ± 1.73	30.0 ± 0.76	28.9 ± 1.14
2	10/22/16 (*n* = 4)	91.8 ± 1.25	29.2 ± 0.22	29.8 ± 1.11
3	10/22/16 (*n* = 4)	88.2 ± 1.50	29.2 ± 0.92	29.9 ± 0.87
4	10/22/16 (*n* = 4)	87.5 ± 1.12	29.8 ± 0.91	32.3 ± 0.81
5	10/23/16 (*n* = 4)	89.3 ± 1.08	30.1 ± 1.02	30.3 ± 0.71
6	10/29/16 (*n* = 4)	89.5 ± 1.11	29.8 ± 1.13	31.0 ± 1.55
7	10/29/16 (*n* = 4)	87.0 ± 1.16	28.8 ± 0.65	28.4 ± 1.81
8	11/3/16 (n = 4)	90.4 ± 0.90	29.5 ± 0.15	30.0 ± 0.88

Yellow-Rumped Warbler cohorts

	Measurement targets:	Wing chord (mm): 70.5	Tarsus (mm): 20.0	Weight (g): 11.5
Cohort #	**Date captured (*n* in cohort)**	**Measured mean ± *SD***	**Measured mean ± *SD***	**Measured mean ± *SD***
1	10/9/15 (*n* = 4)	67.6 ± 0.75	19.9 ± 0.36	11.2 ± 0.51
2	10/11/15 (*n* = 4)	71.0 ± 0.31	20.4 ± 0.09	11.0 ± 1.29
3	10/13/15 (*n* = 4)	68.2 ± 1.44	20.0 ± 0.27	11.8 ± 0.33
4	10/19/15 (*n* = 4)	69.0 ± 0.31	20.2 ± 0.47	12.1 ± 0.48
5	10/19/15 (*n* = 4)	71.0 ± 0.00	20.6 ± 0.38	11.3 ± 0.27
6	11/2/15 (*n* = 4)	71.5 ± 0.00	19.9 ± 0.44	12.0 ± 0.06
7	11/4/15 (*n* = 4)	72.0 ± 1.05	20.1 ± 0.75	11.9 ± 0.40
8	11/4/15 (*n* = 4)	70.5 ± 0.00	20.0 ± 0.00	11.9 ± 0.00

166.38 MHz continuously, allowing birds to be detected when within range (Crewe et al., 2019b). Departure was determined by visually inspecting signal strength in the minutes prior to the tag no longer being detected by either tower on Block Island, following Taylor et al. (2017). In this way a departure event could be distinguished from non-departure events such as an individual losing a tag or the tag becoming inoperable. Briefly, a bird's departure from Block Island was estimated as the time at which a tag's signal strength exhibited a peak (± 8 s), associated with a direct signal from a bird in flight, immediately followed by a rapid decline and loss of the signal as the bird moved out of antenna range (Mitchell et al., 2015; Taylor et al., 2017; Dossman et al., 2018). We were able to further verify that a bird departed the island by determining if it subsequently was detected by a Motus station not on the island. We only included in the analysis of stopover duration and departure direction those birds for which we could confirm departure in this way ($n = 86$).

Analysis of Antioxidant Status

We measured several indicators of antioxidant status and oxidative damage in blood of these birds including non-enzymatic antioxidant capacity (OXY), glutathione peroxidase (GPx, an important enzymatic antioxidant) (Costantini, 2011; Cooper-Mullin and McWilliams, 2016; Cooper-Mullin et al., 2019) and reactive oxygen metabolites (ROMs). Non-enzymatic antioxidant capacity was measured with the OXY-adsorbent test in plasma (5 μL per sample, concentration unit = mmol/L of HClO neutralized; Diacron International, Grosseto, Italy). OXY directly measures the ability of a plasma sample to quench the oxidant hypochlorous acid and provides an index of non-enzymatic antioxidant capacity, without being complicated by inclusion of uric acid (Costantini, 2011; Skrip and McWilliams, 2016). GPx activity in red blood cells was measured indirectly via a coupled reaction with glutathione reductase following the manufacturer's protocol optimized for a small blood volume (1 μL per sample, concentration unit: nmol min^{-1} ml^{-1}; Cayman Chemical glutathione peroxidase assay kit). Oxidized glutathione produced upon reduction of hydroperoxides by GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in A340 is directly proportional to the GPx activity (Celi et al., 2013; Arazi et al., 2017). Oxidative damage was measured using the d-ROMs test (20 μL per sample, concentration unit = mmol/L H_2O_2 equivalents; Diacron International, Grosseto, Italy). This test works by first decreasing the pH of the plasma to release metal ions from proteins to cleave circulating ROMs through incubation with a solution of 0.01 M acetic acid/sodium acetate buffer. The subsequent products react with a chromogen (*N,N*-diethyl-*p*-phenylenediamine) which has a color intensity that is proportional to the concentration of ROMs in the plasma and was measured at 505 nm (Costantini, 2016, 2019). OXY and d-ROMs were measured in duplicate and GPx was measured in triplicate. We only included samples in the analysis if the coefficient of variation was less than 10% (85% of 288 plasma and red blood cell samples).

Statistics

All analyses were done in R version 4.0.2 2021.[1] We used an analysis of variance test (ANOVA) to determine the effect of diet treatments on change in weight, fat score and muscle score during captivity (individual measurement prior to release—measurement at capture) followed by one sample *t*-tests to determine whether this change in weight for maintenance birds of each species was significantly different from zero. For Blackpoll Warbler and Red-eyed Vireo cohorts, we used a one-way ANOVA with two diet treatments (*ad lib*, maintenance) whereas for Hermit Thrush and Yellow-rumped Warbler cohorts, we used a 2 × 2 factorial ANOVA with four diet treatments (*ad lib* or maintenance each with an antioxidant supplemented or not supplemented group).

For each species, we used linear mixed models (*nlme* package)[2] with a fixed effect of treatment group and random effects for cohort and the number of days each bird spent in captivity to examine their effects on how each of the three oxidative parameters changed under each treatment condition: plasma non-enzymatic antioxidant capacity (OXY), GPx activity in red blood cells and plasma oxidative damage (d-ROMs). We analyzed the change in each oxidative measure during captivity (i.e., value prior to release minus that at capture) rather than a one-time measurement on the day of release. Between-individual variation in baseline (capture) measurements of oxidative status can be substantial because of a variety of uncontrolled factors including an individual's diet at its previous stopover site or the type of fat an individual burned during their previous flight. Further, circulating antioxidants may be affected by the amount of circulating oxidative damage or the oxidative status of various tissues. Therefore, the change in oxidative status within an individual during captivity provided us a more robust assessment of the effect of each diet treatment. Cohort was included as a random effect for slope to control for change over the season or environmental conditions during captivity.

We used a linear mixed model with fixed effects for species and treatment group (two diet groups for Blackpoll Warbler and Red-eyed Vireo, four diet groups for Hermit Thrush and Yellow-rumped Warbler) and a random effect of cohort to examine their effects on stopover duration across all species. We also used separate linear or generalized mixed effects models for each species to investigate the effect of the treatment group and change in each oxidative parameter (measurement prior to release—measurement at capture) on stopover duration. Random factors that did not explain any variation in the respective dependent variable were removed from the final model. If no random effect explained any variation, we fit a normal or generalized linear model instead (**Supplementary Table 1**). For all tests, we checked whether data met the assumptions of homogeneity of variance, visually inspected residual plots, and did not find any noticeable deviations from homoscedasticity or normality. We evaluated the fixed effects in each model with likelihood ratio tests and Satterthwaite approximation for degrees of freedom.

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[1] http://www.R-project.org/

[2] https://CRAN.R-project.org/package=nlme

Determining Orientation

We visually inspected plots of signal strength over time from each of the six antennas on each of the two towers to determine when and in which direction each individual departed Block Island. We calculated vanishing bearings for all individuals following the method outlined by Crewe et al. (2019a). We further categorized each vanishing bearing into two categories: "onward migration" to the south and "reverse migration" toward the mainland of Rhode Island. Across all species as well as within each species, we used a mixed effects model with a random effect of cohort to evaluate whether treatment (*ad lib* or maintenance) affected departure direction.

RESULTS

Change in Condition Measures During Captivity

Individual birds spent a median of 3 days in captivity prior to release (**Table 2**, range = 2–6 days for the four species). Change in body mass was influenced by treatment group for Red-eyed Vireos and Blackpoll Warblers [**Figure 2**, $F_{(1,\ 29)} = 37.03$, $P < 0.001$]. Change in body mass was also influenced by treatment group for Hermit Thrushes and Yellow-rumped Warblers [**Figure 2**, $F_{(3,\ 56)} = 19.55$, $P < 0.001$], although *post-hoc* tests revealed that these changes were affected by food availability and not antioxidant treatment (Tukey HSD; *Ad lib*—*Ad Lib* + Dietary Antioxidants: $p = 0.89$, 95% CI = -0.53 to 1.43; Maintenance—Maintenance + Dietary Antioxidants: $p = 0.99$, 95% CI = 0.10–2.00). More specifically, birds fed at maintenance levels did not significantly change body mass during captivity as prescribed (**Figure 2**, Blackpoll Warbler: $t = 5.4$, $P = 0.23$, Red-eyed Vireo: $t = 0.57$, $P = 0.59$, Hermit Thrush: $t = 1.26$, $P = 0.25$, Yellow-rumped Warbler: $t = 1.54$, $P = 0.17$), whereas birds fed *ad lib* significantly increased body mass during captivity (**Figure 2**, Blackpoll Warbler: $t = 5.44$, $P < 0.001$; Red-eyed Vireo: $t = 9.17$, $P < 0.001$; Hermit Thrush: $t = 7.49$, $P < 0.001$; Yellow-rumped Warbler: $t = 6.36$, $P < 0.001$). Birds fed at maintenance levels did not significantly change fat or muscle score during captivity, whereas birds fed *ad lib* increased fat score but not muscle score during captivity (**Supplementary Figure 1**).

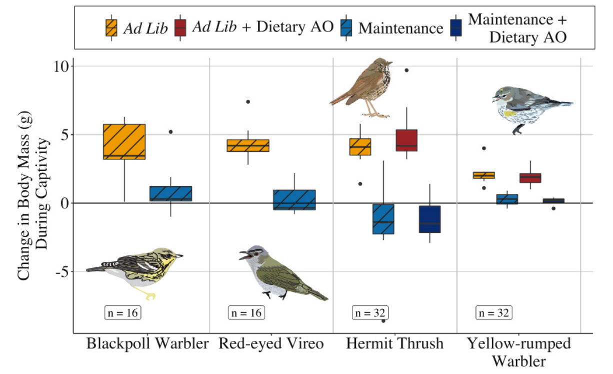

FIGURE 2 | Change in body mass (g) during the ca. 3 days in captivity for Blackpoll Warblers and Red-eyed Vireos given *ad lib* food (gold with diagonal stripes) or maintenance amounts of food (light blue with diagonal stripes), and Hermit Thrushes and Yellow-rumped Warblers given *ad lib* food without and with supplemental antioxidants (gold with diagonal stripes and solid dark red, respectively) or maintenance amounts of food without and with supplemental antioxidants (light blue with diagonal stripes and solid dark blue, respectively). Boxes represent 95% confidence intervals; whiskers represent 1.5 times the interquartile range and outliers are represented by black dots. Labels indicate samples sizes for each species.

Changes in the three measured components of the antioxidant system (i.e., OXY, GPx, d-ROMs) in response to diet treatment are presented for each cohort for each of the four species (**Figures 3**, **4**). Such a presentation emphasizes one of the strengths of the experimental design (i.e., the careful and consistent selection of individuals that were captured on the same day in the same initial condition and of similar body size), it is consistent with the statistical analyses that included cohort as a random effect, and it provides insights into how the different components of the antioxidant system (i.e., OXY, GPx, d-ROMs) changed within and across individuals. Reported in the text here are the treatment effects from each model (**Supplementary Table 1**). Non-enzymatic antioxidant capacity (OXY) increased during captivity for Blackpolls fed *ad lib* compared with Blackpolls fed at maintenance levels (**Supplementary Table 1**, $t = -92.50$, $P = 0.007$, $n = 15$) as well as for Red-eyed Vireos fed *ad lib* vs. maintenance levels (**Supplementary Table 1**, $t = -71.67$, $P = 0.007$, $n = 14$). GPx activity generally decreased during captivity for Blackpolls and we detected no difference between *ad lib* vs. maintenance-fed birds (**Supplementary Table 1**, $t = 5.01$, $P = 0.84$, $n = 10$). For Red-eyed Vireos, GPx activity decreased during captivity for four of the six *ad lib*-fed cohorts and for two of the three maintenance-fed cohorts (**Figure 3D**). On average, maintenance-fed birds decreased GPx activity more than *ad lib*-fed Vireos (**Supplementary Table 1**, $t = -242.60$, $P = 0.02$, $n = 15$). Oxidative damage generally decreased during captivity for both species, although there was no consistent difference in how oxidative damage changed among the treatment groups for Blackpolls (**Figure 3E** and **Supplementary Table 1**, $t = 0.99$, $df = 9$, $P = 0.35$, $n = 14$) or Red-eyed Vireos (**Figure 3F** and **Supplementary Table 1**, $t = 1.56$, $P = 0.21$, $n = 15$).

OXY increased during captivity for almost all Hermit Thrushes and increased more for the thrushes given *ad lib* food with dietary AO (**Figure 4A** and **Supplementary Table 1**, *ad lib* + dietary AO: $t = 4.18$, $P < 0.001$, *ad lib* alone: $t = 1.56$, $P = 0.13$, maintenance + dietary AO: $t = 1.69$, $P = 0.10$; $n = 27$). In contrast, OXY did not significantly change in Yellow-rumped Warblers during captivity for any of the dietary treatments (**Figure 4B** and **Supplementary Table 1**, *ad lib* + dietary AO: $t = 1.03$, $P = 0.31$; *ad lib* alone: $t = -0.11$, $P = 0.92$; maintenance + AO: $t = -0.38$, $P = 0.71$). Most (25 out of 32) Hermit Thrush decreased GPx activity during captivity. However, change in GPx activity during captivity among Hermit Thrush was not consistently different between treatment groups (**Figure 4C** and **Supplementary Table 1**, *ad lib* + dietary AO: $t = 1.40$, $P = 0.17$, *ad lib* alone: $t = 5.92$, $P = 0.91$, maintenance + dietary AO: $t = -0.33$, $P = 0.74$; $n = 27$).

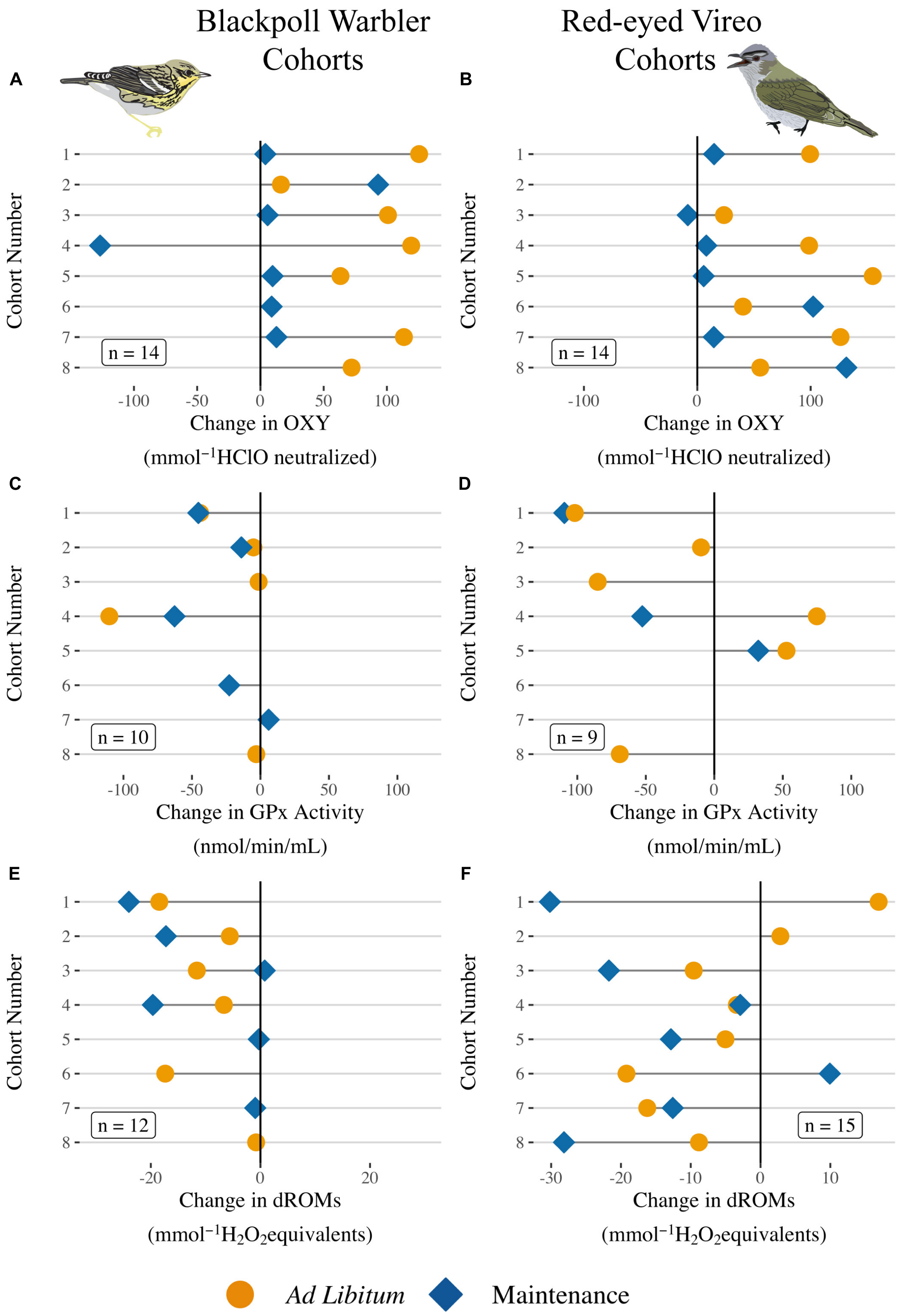

FIGURE 3 | (A,B) Change in plasma non-enzymatic antioxidant capacity (OXY), **(C,D)** erythrocyte glutathione peroxidase activity (GPx), and **(E,F)** plasma oxidative damage (dROMs) for each individual fed *ad lib* (gold circles) or at maintenance levels (light blue diamonds) during captivity by cohort. Cohorts caught later in the season have higher numbers. Each dot represents an individual and the line indicates the magnitude of change during captivity (sample at release—sample at capture). Labels indicate samples sizes for each oxidative measurement per species.

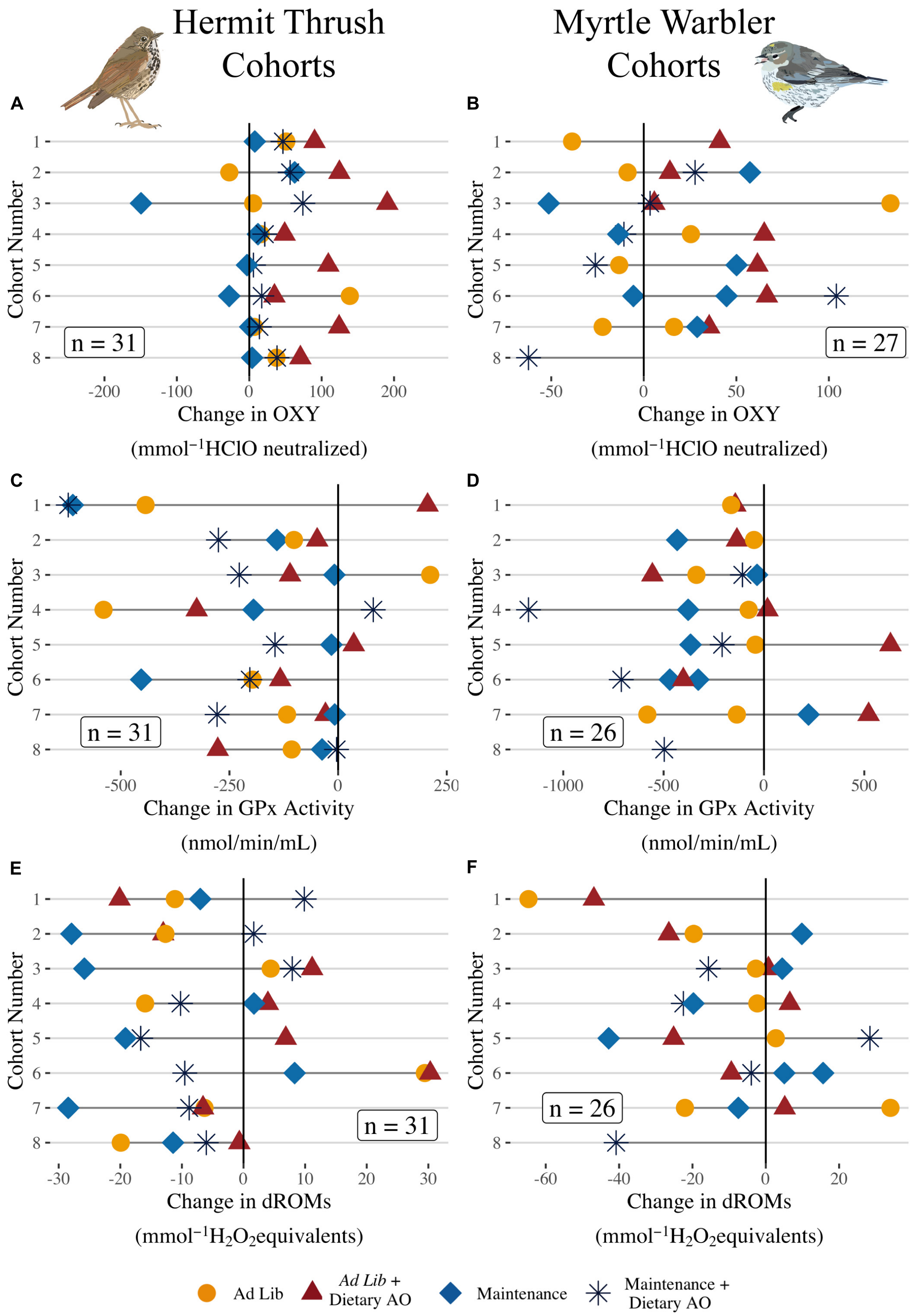

FIGURE 4 | (A,B) Change in plasma non-enzymatic antioxidant capacity (OXY), **(C,D)** erythrocyte glutathione peroxidase activity (GPx), and **(E,F)** plasma oxidative damage (dROMs) for each individual fed *ad lib* without supplemental polyphenols (light red) or with supplemental polyphenols (dark red), or at maintenance levels without supplemental polyphenols (light blue), or with supplemental polyphenols (dark blue) during captivity by cohort. Cohorts caught later in the season have higher numbers. Each dot represents an individual and the line indicates the magnitude of change during captivity (sample at release—sample at capture). Labels indicate samples sizes for each species.

GPx also generally decreased during captivity for Yellow-rumped Warblers and was not significantly different among the treatment groups (**Figure 4D** and **Supplementary Table 1**, $F_3 = 2.41$, $P = 0.09$, $n = 26$). Oxidative damage significantly increased only for thrushes fed *ad lib* food with dietary AO (**Figure 4E** and **Supplementary Table 1**, *ad lib* + dietary AO: $t = 2.43$, $P = 0.02$; *ad lib* alone: $t = 1.74$, $P = 0.083$; maintenance + AO: $t = 1.59$; $P = 0.11$). Oxidative damage did not significantly change in any of the treatment groups for Yellow-rumped Warblers [**Figure 4F** and **Supplementary Table 1**, $F_{(3,\ 20)} = 0.16$, $P = 0.92$, $n = 26$].

Stopover Length and Condition

Blackpoll Warblers fed at maintenance levels and that were lean (**Figure 2** and **Supplementary Figure 1**) departed Block Island later than their *ad lib* fed counterparts [**Figure 5**, stopover duration was 17.38 ± 8.16 vs. 2.32 ± 2.44 days, respectively; $F_{(1,\ 3)} = 86.43$, $P < 0.001$]. Similarly, Red-eyed Vireos fed at maintenance levels and that were lean (**Figure 2** and **Supplementary Figure 1**) departed Block Island later than their *ad lib* fed counterparts [**Figure 5**, stopover duration was 12.81 ± 7.16 vs. 3.24 ± 3.71 days, respectively; $F_{(1,\ 4)} = 4.82$, $P = 0.02$]. Blackpoll Warblers fed at maintenance levels and Red-eyed Vireos fed at maintenance or *ad libitum* levels that were able to increase OXY more during captivity departed sooner than birds with lower OXY [**Figure 6**; $F_{(1,\ 3)} = 23.81$, $P = 0.02$ and $F_{(1,\ 6)} = 6.21$, $P = 0.03$, respectively]. When we re-ran the analysis without the maintenance-fed Blackpoll Warbler that remained on Block Island for 32 days, Blackpoll Warblers that were able to increase OXY during captivity across both treatment groups were able to depart Block Island sooner than those individuals that were not able to increase their non-enzymatic antioxidant capacity [$F_{(1,\ 11)} = 5.36$, $P = 0.04$]. Change in OXY during captivity was not associated with stopover length in Hermit Thrush [$F_{(1,\ 20)} = 1.61$, $P = 0.22$] or Yellow-rumped Warblers [$\chi^2 = 0.07$, $P = 0.79$]. Hermit Thrushes fed at maintenance levels had longer stopovers than those fed *ad lib* (**Figure 5**, $t = -6.60$, $P < 0.001$), and those fed at maintenance levels with supplemental AO had shorter stopover lengths than those fed at maintenance levels without supplemental AO (maintenance + AO: 9.72 ± 3.96; maintenance: 16.27 ± 3.85). Thrushes fed an *ad lib* diet supplemented with dietary AO had the shortest stopover lengths (*ad lib* + AO: 5.81 ± 0.82; *ad lib*: 6.78 ± 0.88). There was no effect of diet treatment on the number of days Yellow-rumped Warblers spent on stopover (maintenance: 17.97 ± 10.07, maintenance + dietary AO: 18.61 ± 6.83, *ad lib*: 13.45 ± 10.35, *ad lib* + dietary AO: 18.54 ± 6.00, $\chi^2 = 0.53$, $P = 0.47$). Across species, Red-eyed Vireos fed at maintenance levels had shorter stopover durations than Blackpoll Warblers fed at maintenance levels ($P = 0.02$), and Yellow-rumped Warblers had on average the longest stopover durations ($P = 0.04$).

Departure Direction

We were able to obtain vanishing bearings for 14 Blackpoll Warblers, 14 Red-eyed Vireos, 31 Hermit Thrushes, and 27 Yellow-rumped Warblers (**Figure 7**). Departure direction was not influenced by feeding treatment group ($\chi^2 = 0.33$, $P = 0.56$), and the majority of birds departed in a reverse migratory direction (reverse migration: $\chi^2 = 11.51$, $P < 0.001$). Specifically, nine (75%) Blackpoll Warblers, 8 (67%) Red-eyed Vireos, 20 (69%) Hermit Thrushes, and 15 (68%) Yellow-rumped Warblers departed Block Island heading back to the mainland of Rhode Island.

DISCUSSION

Our short-term field experiments with four species of free-living, migrating songbirds revealed that for 3 of the 4 species included, birds with enhanced fat stores and non-enzymatic antioxidant capacity departed sooner from a stopover site, and that the extent of this condition-dependent behavior was related to a species migratory strategy. Specifically, hatch-year Hermit Thrush, Red-eyed Vireos, and Blackpoll Warblers fed *ad lib* for several days in captivity had more substantial fuel stores and non-enzymatic antioxidant capacity and departed from an offshore stopover site at least two times faster than maintenance-fed, leaner birds. A similar response was not observed for Yellow-rumped Warblers, a shorter-distance migrant. We also found that songbirds decreased enzymatic antioxidant capacity during rest and recovery on stopover. These results indicate that an individual's fat stores as well as antioxidant capacity can act as endogenous cues to continue migration.

> ***Hypothesis 1: Circulating antioxidant capacity changes with rest and refueling on stopover and the extent and direction depends on availability of dietary antioxidants and the component of antioxidant capacity measured***

Non-enzymatic Antioxidant Capacity

During short-term captivity, *ad lib*-fed Blackpoll Warblers and Red-eyed Vireos increased their fat stores and non-enzymatic antioxidant capacity more than individuals of the same species fed at maintenance levels, indicating that food availability on stopover allows individuals to increase non-enzymatic antioxidant capacity. Although the exact mechanism is unknown, it is likely that increased food availability on stopover leads to higher levels of non-enzymatic antioxidant capacity either directly via dietary antioxidants (DeMoranville et al., 2021; McWilliams et al., 2021) or through upregulation of endogenous antioxidants to combat an increase in RS production from lipid peroxidation (Skrip and McWilliams, 2016). These results confirm previous work from Block Island that found fat stores were positively correlated with non-enzymatic antioxidant capacity in free-living Red-eyed Vireos and Blackpoll Warblers (Skrip et al., 2015). In addition, fasting-refeeding trials in captive Northern Wheatears indicated that fueling boosted total non-enzymatic antioxidant capacity (as measured by ferric reducing antioxidant power), although those results were primarily attributed to increased circulating uric acid (Eikenaar et al., 2016a). Since the OXY-adsorbent test excludes uric acid, our results indicate that Blackpoll Warblers and Red-eyed Vireos were able to increase other important circulating non-enzymatic antioxidants (e.g., other sacrificial

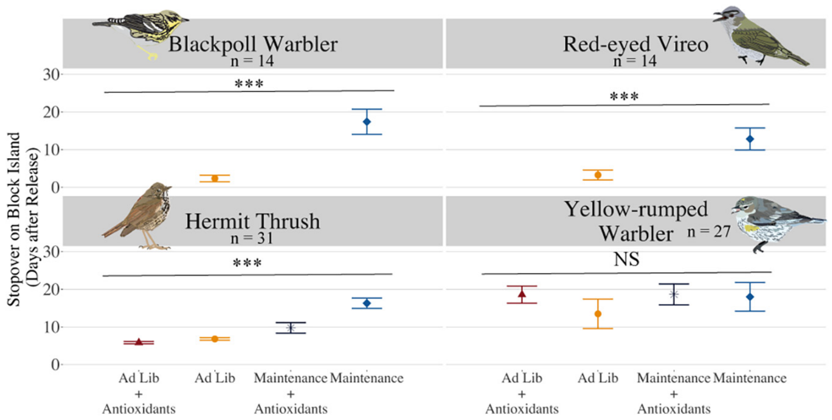

FIGURE 5 | Stopover duration (days on Block Island after release from captivity) for individuals fed *ad lib* without supplemental polyphenols (gold circles) or with supplemental polyphenols (dark red triangles), or at maintenance levels without supplemental polyphenols (light blue diamonds), or with supplemental polyphenols (dark blue stars). “***” indicates that $P < 0.001$.

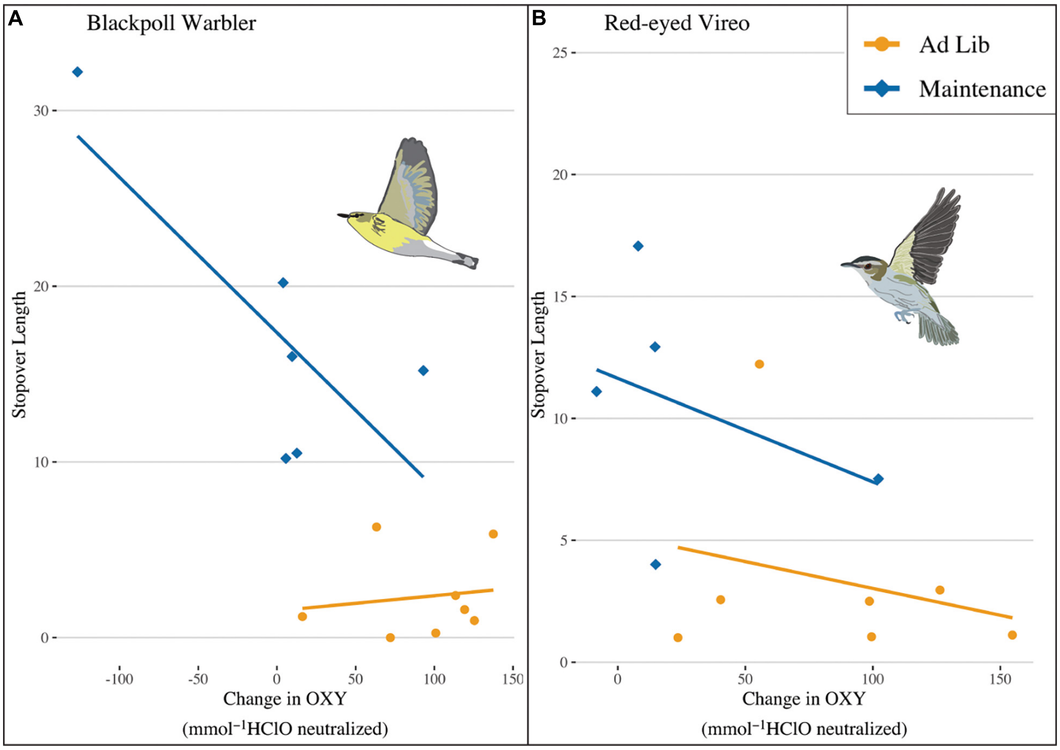

FIGURE 6 | Relationship between non-enzymatic antioxidant capacity during captivity (blood sample prior to release—blood sample at capture) and days spent on stopover after release for **(A)** Blackpoll Warblers fed *ad lib* (gold circles) or at maintenance levels (light blue diamonds). **(B)** Red-eyed Vireos fed *ad lib* (gold circles) or at maintenance levels (light blue diamonds). There were no significant relationships for Hermit Thrush or Yellow-rumped Warblers (not shown, see text for details).

molecules, precursors for enzymatic antioxidants) while building fat stores (Tsahar et al., 2006; Costantini, 2011; Carro et al., 2012; Skrip and McWilliams, 2016).

When we provided short-term captive Hermit Thrushes with supplementary hydrophilic antioxidants (i.e., daily doses of polyphenols), all birds increased OXY during captivity, and those fed *ad lib* and provided polyphenols were able to increase OXY more than any other treatment group. This indicates that hydrophilic antioxidants, such as those abundant in most fruits on Block Island and elsewhere (Schaefer, 2011; Alan et al., 2013; Bolser et al., 2013), are able to directly influence an individual’s non-enzymatic antioxidant capacity. Interestingly, Blackpolls and Vireos given an *ad lib* diet were also able to increase their non-enzymatic antioxidant

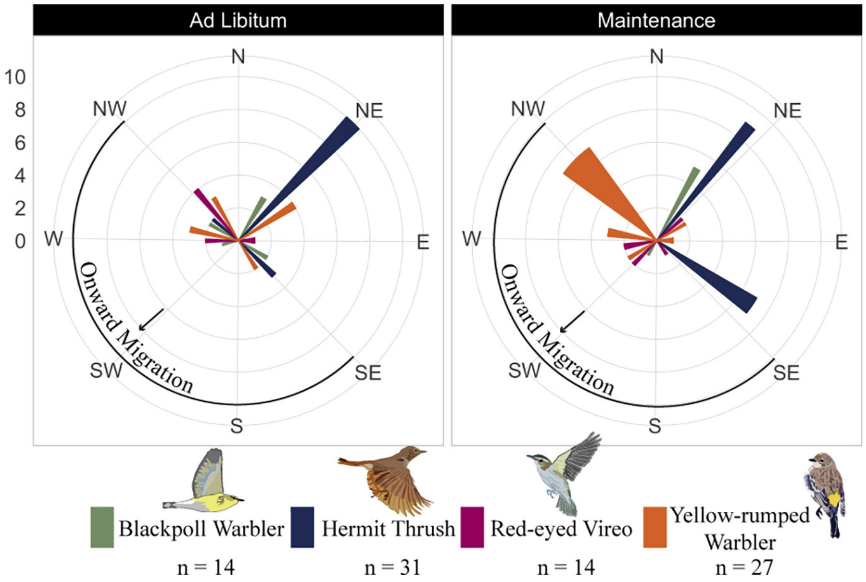

FIGURE 7 | Departure directions for fall-migrating Blackpoll Warblers (green), Hermit Thrushes (blue), Red-eyed Vireos (pink), and Yellow-rumped Warblers (yellow) fed *ad lib* or at maintenance levels. Departure decisions (shaded directions are onward, unshaded directions are reverse) were not influenced by feeding treatment, and the majority of birds departed in a reverse migratory direction to the north from their stopover on Block Island, RI, United States.

capacity above those birds fed at maintenance levels and without supplementary dietary antioxidants. Since waxworms can have up to 69.2 mg/kg of vitamin E (Finke, 2015), it seems possible this lipophilic antioxidant was responsible for increased non-enzymatic antioxidant capacity for these *ad lib* fed birds. Hermit Thrushes fed at maintenance levels and supplemented with dietary antioxidants had comparable levels of non-enzymatic antioxidants to those fed *ad lib* and without additional antioxidants. This confirms that availability of hydrophilic antioxidants can increase non-enzymatic antioxidant capacity in migratory songbirds such as Hermit Thrushes. On stopover in the fall, birds primarily forage on locally abundant fruits, but not all fruits are high in both fat and antioxidant content (Benvenuti et al., 2004; Alan et al., 2013; Bolser et al., 2013; Schaefer et al., 2014). These results also suggest that consuming fruits with low fat content but sufficient antioxidants, such as Winterberry (*Ilex verticillata*; Alan et al., 2013), while on stopover may still allow birds to increase non-enzymatic antioxidant capacity, but in different ways. However, further research is needed on the absorption (Beaulieu and Schaefer, 2013; Skrip and McWilliams, 2016), possible microbial contribution (Laparra and Sanz, 2010; Saag et al., 2011), and the integration of dietary antioxidants into other physiological systems (Aboonabi and Singh, 2015; Hamilton et al., 2018; DeMoranville, 2020) in migratory birds for an adequate understanding of how dietary antioxidants improve antioxidant capacity.

In contrast, Yellow-rumped Warblers did not increase non-enzymatic antioxidant capacity even when supplemented with dietary antioxidants. There are three possible, non-mutually exclusive, explanations for why Yellow-rumped Warblers responded differently to our dietary treatments than Hermit Thrushes, Blackpoll Warblers, and Red-eyed Vireos. First, Yellow-rumped Warblers have specialized digestive enzymes that allow them to digest the waxy bayberry fruits (Place and Stiles, 1992; Afik et al., 1995). It is possible that this adaptation has changed how Yellow-rumped Warblers absorb and metabolize dietary antioxidants. Second, Yellow-rumped Warblers may rely more heavily on other aspects of their antioxidant system (e.g., enzymatic antioxidants) to combat damage during migration (Cooper-Mullin and McWilliams, 2016). Third, these warblers are shorter-distance migrants that winter relatively farther north than the other species in this study, and thus they may not need to increase non-enzymatic antioxidant capacity to the same extent as the other three species (Metzger and Bairlein, 2011). Therefore, the degree to which birds increase non-enzymatic antioxidant capacity while refueling may be linked to their migratory strategy, distance to migratory destination, and species-specific physiology.

Enzymatic Antioxidant Capacity (Glutathione Peroxidase)

For all four species, GPx activity decreased during short-term captivity indicating that birds downregulate enzymatic antioxidant capacity while resting on stopover. Further, change in GPx activity was unaffected by diet treatment, indicating that enzymatic antioxidants are physiologically regulated separately from non-enzymatic antioxidant capacity during stopover. These results are in line with the one other study that examined the effect of rest on circulating GPx activity during migration (Jenni-Eiermann et al., 2014). Free-living European Robins (*Erithacus rubecula*) caught while resting and refeeding at a stopover site exhibited the highest concentrations of GPx at dawn, presumably soon after setting down from a migratory flight, and GPx activity decreased throughout the day as robins rested and refueled regardless of fat score at capture (Jenni-Eiermann et al., 2014). Further, in a captive experiment, individual Zebra Finches rapidly upregulated GPx in response to acute exercise training yet returned to their baseline GPx activity by the next day (Cooper-Mullin et al., 2019). Therefore, it is likely that this enzymatic antioxidant acts as a first line of defense for responding to the oxidative challenges of flight, but that perhaps due to the ubiquitousness of GPx across body tissues (Halliwell and Gutteridge, 2007; Margis et al., 2008; Cooper-Mullin and McWilliams, 2016), maintaining high levels of circulating GPx is too costly during periods of rest when production of RS is expected to be low.

Hypothesis 2: Oxidative damage decreases with rest and refueling on stopover but the extent likely depends on fat stores and availability of dietary antioxidants

Although all birds fed *ad lib* in captivity increased fat stores, the relationship between fat and oxidative damage across species was varied. Blackpoll Warblers decreased oxidative damage during captivity, although those fed *ad lib* decreased oxidative damage less than those fed at maintenance levels. In contrast, oxidative damage decreased for Red-eyed Vireos during the few days in captivity regardless of treatment group. The only treatment group to notably increase oxidative damage during captivity was Hermit Thrushes given an *ad lib* diet supplemented with dietary antioxidants. It is possible that the incorporation of hydrophilic antioxidants from the diet interfered with the upregulation or circulation of lipophilic antioxidants to combat lipid damage (Skrip et al., 2016); clearly, more research on the integration of different aspects of the antioxidant system in migratory birds are needed. A decrease in circulating lipid hydroperoxides during captivity provides evidence that songbirds can recover from oxidative damage after flight, but it is important to note that our measure of oxidative damage may be the product of RS accumulated during flight, consumption of oxidatively-vulnerable fats during stopover, and the relative activity of repair mechanisms as well as the antioxidant system (Halliwell and Gutteridge, 2007; Cooper-Mullin and McWilliams, 2016). It is possible that increased levels of circulating fats prevented *ad lib* fed Blackpolls from reducing circulating oxidative damage as much as maintenance fed Blackpolls. Additionally, *ad lib* fed thrushes given dietary antioxidants increased ROMs, but also had the highest circulating levels of OXY. These results along with those reported by Skrip et al. (2015) and Eikenaar et al. (2016a) imply that circulating oxidative damage to lipids may decline as individuals build fat stores at stopover sites, but the extent depends on other ecologically relevant factors including diet quality as well as the intensity of the previous flight and the antioxidant system response.

Hypothesis 3: Individuals that are able to build non-enzymatic antioxidant capacity and/or fuel stores (i.e., in better body condition) on stopover are able to depart more quickly and are less likely to exhibit reverse migration

We experimentally demonstrated that Blackpoll Warblers, Red-eyed Vireos and Hermit Thrushes fed *ad lib* and thus with greater fuel stores departed an offshore stopover site more quickly than their maintenance-fed counterparts. These results generally agree with the other two single-species studies that have experimentally manipulated fuel stores of migrating songbirds on stopover sites (Smith and McWilliams, 2014; Dossman et al., 2018). Individuals on stopover with sufficient fuel stores are more likely to leave as soon as weather is favorable for migration (Schmaljohann et al., 2012; Smith and McWilliams, 2014). Past studies at other coastal stopover sites have shown that if birds lose energy stores or refuel at slower rates, as would occur at low-quality stopover sites, then the probability of departure from the stopover site was higher than that of birds refueling at higher rates (Schaub et al., 2008; Aamidor et al., 2011; Morganti et al., 2011; Schmaljohann et al., 2013; Paxton and Moore, 2017). Given that maintenance-fed Blackpoll Warblers, Red-eyed Vireos and Hermit Thrushes spent more time on Block Island than their *ad lib*-fed counterparts, presumably taking advantage of Block Island's abundant fruiting plants for refueling, these results indicate that Block Island is apparently a higher quality stopover site.

Ours is the first study to demonstrate that non-enzymatic antioxidant capacity can directly influence stopover duration in long- and short-distance migratory songbirds. Hermit Thrushes that did not receive supplementary antioxidants had longer stopovers compared with those that consumed more dietary antioxidants. This effect of dietary antioxidants was most pronounced when birds were fed at maintenance levels indicating that availability of dietary antioxidants, independent of fat deposition, can influence stopover duration. Further, Red-eyed Vireos that were able to increase their non-enzymatic antioxidant capacity during captivity had shorter stopovers than those that did not, as did maintenance-fed Blackpoll Warblers. Therefore, increased non-enzymatic antioxidant capacity seems to provide migratory birds with an important cue that, in addition to fat stores, influences departure decisions from stopover sites, although more research on the mechanisms and molecular underpinnings of these signals is needed.

In contrast to the other three species, we saw no impact of diet quality and quantity on stopover duration of Yellow-rumped Warblers. Yellow-rumped Warblers are unusual compared to the other three focal species in that they have a widely available diet source (bayberry) on Block Island and other coastal stopover sites that only they can digest (Place and Stiles, 1992; Podlesak et al., 2005; Bolser et al., 2013). Thus, after release from our short-term captive experiments, regardless of diet treatment, Yellow-rumped Warblers may have been able to fuel relatively quickly, and this may essentially erase any short-term condition differences that we created with our diet quantity and quality manipulations. Yellow-rumped Warblers were also likely near the end of their migratory journey once they reach New England (Murray and Murray, 1979), although clear understanding of the migratory patterns among different populations of this species remains murky. Therefore, minimizing time on stopover may not be as important for Yellow-rumped Warblers as conserving energy (Hedenström and Alerstam, 1997) compared to the other three species.

If individuals that build high non-enzymatic antioxidant capacity can leave stopover faster, stopovers with fruits that are high in both fats and antioxidants represent extremely valuable resources for Neotropical and other frugivorous migrants (Alan et al., 2013; Bolser et al., 2013; Smith et al., 2013). There is evidence that birds may be able to distinguish the antioxidant content of fruits (Schaefer et al., 2008, 2014) and previous work on Block Island demonstrated that passerines preferentially consume fruits with higher fat and antioxidant content (e.g., arrowwood, Alan and McWilliams, 2013; Bolser et al., 2013). Such preferences for certain fruits have important ecological implications in that non-native or invasive plant species produce fruits that in general are less nutritious though abundant (Drummond, 2005; Mudrzynski and Norment, 2013; Smith et al., 2013; Smith and Hatch, 2017), although too little information is available on the antioxidants in fruits of non-native species.

For all four focal species in our study, the majority of individuals departed Block Island heading north and thus reoriented back to the mainland. Formative work by Able (1977) with Blackpoll Warblers on Block Island also demonstrated that birds departing stopover overwhelmingly reoriented back to the mainland. Further, for birds in our study, orientation of departure flight was not associated with condition. This contrasts with a previous experiment on Block Island that found Hermit Thrushes fed *ad lib* had a higher likelihood of continuing migration in a seasonally appropriate southerly direction (Smith and McWilliams, 2014). As fruit resources on Block Island can vary from year to year, as well as within a season, it is possible that the distribution of food on Block Island between years influenced Hermit Thrush departure orientation (Smith et al., 2007; Bolser et al., 2013; Smith and McWilliams, 2014).

In the Gulf of Maine, Blackpoll Warblers and Red-eyed Vireos often leave coastal stopovers by orienting inland (Brown and Taylor, 2017; Smetzer and King, 2018). However, those movements are often explained as birds fleeing poor refueling habitat at coastal sites (Mehlman et al., 2005; Woodworth et al., 2015). Since our experimental manipulations produced birds with more fat stores, and Block Island offers good habitat for refueling (Reinert et al., 2002; Smith and McWilliams, 2010; Skrip et al., 2015), there are likely other drivers of reorientation behavior for these species. Most birds that stopover on Block Island are hatch year individuals that presumably were displaced offshore on nights with strong northwest winds, so birds that reorient may be retracing their steps back to familiar habitat before continuing migration (Baird et al., 1959; Able, 1977; Åkesson et al., 1996; Mitchell et al., 2011). Although further research is necessary to determine whether individuals departing Block Island on a given night were exhibiting true migratory movement or were exhibiting extended stopover or landscape scale movement (Mills et al., 2011; Taylor et al., 2011; Brown and Taylor, 2017), initial departure direction was not influenced by condition.

Hypothesis 4: Species that migrate farther, or those that are more likely to undertake a long-distance flight exhibit a different degree of condition-dependent behavior while on stopover

Blackpoll Warblers, Red-eyed Vireos, and Hermit Thrushes fed at maintenance levels stayed on Block Island up to 6, 3, and 2 times longer, respectively, than those fed *ad lib*. However, Yellow-rumped Warblers had similar stopover durations regardless of treatment group. Therefore, departure decisions were condition-dependent for 3 out of the 4 species we studied, and the extent of condition-dependent effects on stopover duration may be related to differences in migratory strategies. For example, Red-eyed Vireos fed at maintenance levels departed from Block Island faster than maintenance-fed Blackpoll Warblers, possibly because Blackpoll Warblers face a greater geographical barrier (the ocean) upon departure from Block Island (DeLuca et al., 2015, 2019). Many Blackpoll Warblers initiate a trans-Atlantic crossing from New England and fly non-stop over the North Atlantic Ocean to the Caribbean or South America (Nisbet, 1970; DeLuca et al., 2015, 2019), a journey that requires at least 10–12 g of fat stores (Klaassen, 1996; Boal, 2014). Among Blackpolls measured at three coastal stopover sites and nine inland stopover sites during migration, individuals on Block Island were the heaviest and had the largest fat stores (Morris et al., 2016), emphasizing the importance of this stopover for Blackpolls. Additionally, the stopover duration measured in our experimental birds was within the range of other estimates of stopover for fall-migrating birds on Block Island that were simply captured, banded, and recaptured (Parrish, 2000). Thus, condition-dependent stopover behavior is common for migratory passerines (except Yellow-rumped Warblers) and there is variation in the extent to which condition may drive behavioral decisions that seems related to the migration strategy of the species.

Yellow-rumped Warblers had the longest stopovers of all species studied and may respond to fuel stores similarly to other medium-distance migrants. For example, there was no relationship between fuel deposition rate and departure fuel load in European Robins, a short-to-medium distance migrant, provided with *ad lib* mealworms at a stopover site in Sweden (Dänhardt and Lindström, 2001). Although Yellow-rumped Warblers and Hermit Thrush have similar migration travel distances, the wintering ecology of these species may shape time constraints on stopover (Kwit et al., 2004; Alvarado et al., 2014; Hunt and Flaspohler, 2020). Hermit Thrush often set up territories on their wintering grounds and individuals that arrive first choose the highest quality areas (Kwit et al., 2004). In contrast, Yellow-rumped Warblers usually forage together in flocks on the wintering grounds, a much less competitive lifestyle (Kwit et al., 2004). Therefore, Hermit Thrushes may be more time constrained on migration than Yellow-rumped Warblers.

Stopover sites (e.g., Block Island) are crucial for birds recovering from the physiological challenges of migratory flight (Ktitorov et al., 2010; Chernetsov, 2012; Smith et al., 2015) and here we demonstrated that the quality of resources available affects how long birds remain at a stopover site. This multi-species study provides direct experimental evidence for how the ability to rebuild fuel and antioxidant stores at a stopover can directly influence the pace of migration by influencing the time an individual spends at a stopover site, and how these responses vary across species with different migratory challenges. What remains to be determined is how variation in fruit species and availability across stopover sites may alter these decisions, how decisions at one stopover location may carryover to decisions at the next (Legagneux et al., 2012; McKinnon et al., 2015; Skrip et al., 2016). Also unknown is (1) exactly how absorption of dietary antioxidants and fats from fruits are affected by the physiological state of the organism (e.g., gut microbiome; Trevelline et al., 2019) or by other macro-and-micronutrients in the fruits and (2) how antioxidants consumed at a stopover site are integrated into other physiological systems (e.g., peroxisome proliferator-activated receptors, glucocorticoid production Hamilton et al., 2018; Casagrande et al., 2020). The results from our experiment can help inform future studies that examine whether the behavioral responses of these four species at one stopover site do in fact influence the overall pace and survival of birds during migration. Future studies should examine the carry-over effects of fueling and antioxidant status from one stopover site to the next, and how this relates to arrival times and behavior at wintering grounds.

DATA AVAILABILITY STATEMENT

The data underlying the results presented in the study are available from Dryad: https://doi.org/10.5061/dryad.9w0vt4bhh.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Rhode Island Office of Research Integrity.

AUTHOR CONTRIBUTIONS

SM and CC-M: conceptualization, writing—review and editing, and visualization: CC-M: writing—original draft preparation. SM: funding acquisition, resources, and supervision. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.762146/full#supplementary-material

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Early and Late Migrating Avian Individuals Differ in Constitutive Immune Function and Blood Parasite Infections – But Patterns Depend on the Migratory Strategy

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Billions of birds migrate every year. To conduct a successful migration, birds undergo a multitude of physiological adaptions. One such adaptation includes adjustments of immune function, however, little is known about intraspecies (between-individual) and interspecies (between-species) variation in immune modulations during migration. Here, we explore if early and late migrating individuals differ in their immune function, and if such patterns differ among species with short- vs. long-distance migration strategies. We quantified three parameters of baseline (constitutive) innate immune function and one parameter of baseline (constitutive) acquired immune function in 417 individuals of 10 species caught during autumn migration at Falsterbo (Sweden). Early and late migrating individuals differed in lysis and total immunoglobulins (IgY), but the patterns show different directions in long-distance migrants (LDMs) (wintering in Africa) as compared to short-distance migrants (SDMs) (wintering within Europe). Specifically, early migrating LDMs had lower lysis but higher immunoglobulin levels than late migrating individuals. In short distance migrants, there was no difference in lysis between early and late migrating individuals, but immunoglobulin levels were higher in late migrating individuals. We found no correlation between timing of migration and haptoglobin, but LDMs had lower levels of haptoglobin than SDMs. We also found that the prevalence of haemosporidian blood parasite infections decreased in LDMs, but increased in SDMs, as the autumn progressed. Taken together, our study suggests that the investment into immune function depends on the migratory strategy (short- vs. long-distance migrants), and that early and late migrating individuals of a migration strategy might invest differently in baseline immune function, potentially driven by differences in the trade-offs with timing and speed of migration. Our study highlights the potential adaptations of immune function that could help explain trade-offs with other physiological systems, and behavioural responses during migration.

Keywords: optimal migration theory, ecophysiology, avian migration, stopover ecology, eco-immunology, migratory strategy, avian malaria

INTRODUCTION

Each year billions of birds migrate from breeding to non-breeding grounds. The timing of migration, as well as the distance covered between the breeding and the wintering grounds, differs greatly both among and within species. Species migrating to winter in sub-Saharan Africa typically migrate in early autumn and are often referred to as long-distance migrants (LDMs) (Berthold, 1993; Newton, 2008). In contrast, species migrating from Northern Europe to winter in western or southern Europe are often referred to as short-distance (or medium-distance) migrants and typically migrate later during the autumn season (Berthold, 2001; Newton, 2008). LDMs are hypothesised to be time-minimisers, selected to arrive at the wintering grounds as early as possible (Alerstam and Lindström, 1990; Hedenström and Alerstam, 1997; Alerstam et al., 2003). On the other hand, short-distance migrants (SDMs) are more likely to be energy-minimisers (Alerstam and Lindström, 1990; Hedenström and Alerstam, 1997). During migration, birds need to adjust their physiology to a multitude of challenges, including but not limited to environmental changes and sustained periods of extreme physical exertion (Piersma and van Gils, 2011). During the last decades, studies on the physiological adjustments required during migration have focused on energetic aspects (and hormonal adjustments) (Blem, 1980; Alerstam and Lindström, 1990; Gwinner, 1990; Lindström and Alerstam, 1992; Lindström, 2003; Guglielmo, 2010; Piersma and van Gils, 2011). However, recent studies indicate that there are also a number of other physiological systems adjusted during migration (Piersma and van Gils, 2011; Cooper-Mullin and McWilliams, 2016; Hegemann et al., 2019; McWilliams et al., 2021). In particular, some studies have highlighted that migration is connected with changes in the immune system that can influence migratory behaviour and processes. For example, both constitutive immune function and responsiveness to immune challenges can be reduced during the migratory season (Owen and Moore, 2006, 2008a,b; Buehler et al., 2008b, 2010b; Eikenaar and Hegemann, 2016). Also trade-offs between immune function and other physiological systems like oxidative balance may occur during migration (Eikenaar et al., 2018) and stopovers may not only be required for refuelling, but also for recovery of immune function (Owen and Moore, 2008b; Buehler et al., 2010b; Eikenaar et al., 2020a,b). Furthermore, levels of constitutive immune function relate to stopover duration (Hegemann et al., 2018b) and to the length of migration delays if individuals need to mount an immune response during the migratory season (Hegemann et al., 2018a). However, many questions regarding these physiological adaptations still remain open (Schmaljohann and Eikenaar, 2017; Hegemann et al., 2019).

The immune system protects the body from pathogens and other non-self particles and is therefore important for survival (Roitt et al., 1998), but at the same time, it incurs costs in terms of production, maintenance and activation (Klasing, 2004; Hasselquist and Nilsson, 2012). Several studies found evidence for downregulation of (parts of) constitutive immune function during migration (Owen and Moore, 2006; Buehler et al., 2010b; Eikenaar and Hegemann, 2016), presumably resulting from resource or risk trade-offs (Owen and Moore, 2008b; Buehler et al., 2010b; Eikenaar et al., 2020a). However, information whether there are general differences between species with different migratory strategies [e.g., long-distance vs. short-distance migrants (SDMs)] is currently lacking. Migratory strategies may also lead to different trade-offs with the demands for migration itself, resulting in differential investment in immune function (Hasselquist, 2007; Buehler and Piersma, 2008; Buehler et al., 2010a). In particular, LDMs have been hypothesised to have a higher susceptibility to infectious diseases, because the high physiological demands of long-distance migration may lead to reduced immune function (Klaassen et al., 2012). Furthermore, both the timing and the destination of migration may influence the risk of exposure to pathogens encountered by the migrants (Altizer et al., 2011; Hall et al., 2016). For example, many infectious diseases as well as vectors of pathogens show seasonal patterns and hence early migrating individuals and species may be exposed to other disease risks than late migrating species or individuals (Altizer et al., 2006; van Dijk et al., 2014), and tropical areas are supposed to harbour more pathogens than temperate regions (Altizer et al., 2011; Westerdahl et al., 2014). Finally, within species there is considerable variation in timing of migration, which may result in individual differences in trade-offs between immune function and migration. Yet, comprehensive data on seasonal patterns of immune function among long- and short-distance migrants, as well as early and late migrating individuals within a species, are lacking. Such knowledge would also help to understand disease transmission patterns and to identify the time windows with the highest risk of spreading of disease (Owen et al., 2021).

A recent meta-analysis of published papers on infection status and infection intensity in migratory hosts found that across animal taxa, parasites have a negative impact on migration performance and that migratory performance decreased with increasing infection intensity (Risely et al., 2018). Hence, infected birds migrate more slowly and may therefore lag behind conspecifics. Yet, data on temporal segregation of infected birds across a migratory season are still rare. An analysis of four passerine species on spring migration revealed that late migrating individuals had a much higher prevalence of haemosporidian blood parasites [one of the most common chronic infections in birds (Valkiūnas, 2004)] than early migrating individuals (Emmenegger et al., 2018). In Yellow-rumped Warblers *Dendroica coronate* during spring migration, both the probability and the intensity of blood parasite infections were higher for later migrating birds (DeGroote and Rodewald, 2010). Both studies suggest that blood parasite infections may hamper spring migration (see also Garvin et al., 2006) or that reduced feather growth during moult in infected individuals may delay the onset of spring migration (see Marzal et al., 2013). Indeed, during autumn migration, SDMs infected with avian haemosporidians had three times as long stopover periods as uninfected birds, though there was no difference in stopover duration between infected and uninfected LDMs (Hegemann et al., 2018b). However, that study did not investigate effects of seasonal timing. To our knowledge, the only study investigating seasonal patterns of blood parasite infections in autumn migrating birds found that late migrating European Robins *Erithacus rubecula* tended to have higher prevalence of avian malaria than early migrating individuals (Agh et al., 2019). Hence, comprehensive data on temporal differences between infected and non-infected individuals during autumn migration are still missing.

In this study, we investigated if LDMs and SDMs differ in baseline (constitutive) immune function and if, within either group, early-migrating individuals differ from late migrating individuals. We sampled 417 individual birds of 10 species during autumn migration stopover at Falsterbo (Sweden). Our aims were to quantify (1) if early migrating individuals (within a species) have different immune function or different haemosporidian prevalence compared to late migrating individuals, and (2) if these patterns differ between LDMs and SDMs. We hypothesised that due to stronger selection for time-minimisation, late LDMs would have lower baseline immune function compared to early migrating individuals as they need to invest more resources in fast migration. In contrast, we did not expect any differences in immune function between early and late migrants among SDMs as all of them were relatively close to their final destination and therefore not time-stressed. Regarding blood parasite infections, following earlier work during spring migration, we expected late individuals to have higher prevalence of blood parasites than early migrating individuals independent of the migration strategy.

MATERIALS AND METHODS

We sampled passerine species during the 2014 autumn migration season at the Falsterbo Peninsula, a strategic stopover site in Southwest Sweden (55.383°N, 12.816°E). Birds were caught as part of a standardised ringing project in the lighthouse garden assumed to be migrants during stopover (Karlsson and Bentz, 2004; Hegemann et al., 2018a,b). We sampled birds between 26 August and 11 November. Birds were caught between sunrise and 12:45 h CET and mistnets (maximum of 21 nets of 9 m each; 16 mm mash size, see Karlsson and Bentz, 2004) were checked every half hour and birds were brought for further handling into the ringing hut. Birds were aged based on plumage characteristics (Svensson, 1992) and we included only hatch-year birds (which constitute about 90% of all ringed birds at Falsterbo; Falsterbo Bird Observatory, unpublished data) in our study. All species of which less than 10 individuals were sampled were excluded, resulting in samples from 417 individuals of 10 species. Six of those species (Blackbird *Turdus merula*, $n = 15$, Blue Tit *Cyanistes caeruleus*, $n = 38$, Chaffinch *Fringilla coelebs*, $n = 24$, Dunnock *Prunella modularis*, $n = 63$, Robin *E. rubecula*, $n = 98$, and Song Thrush *Turdus philomelos*, $n = 62$) are SDMs that winter in Western and Southern Europe, and four species (Common Redstart *Phoenicurus phoenicurus*, $n = 33$, Garden Warbler *Sylvia borin*, $n = 20$, Tree Pipit *Anthus trivialis*, $n = 27$, Willow Warbler *Phylloscopus trochilus*, $n = 37$) are LDMs that winter in sub-Saharan Africa.

We collected blood samples (~100 μl; always less than 10% of an individual's total blood volume) by puncturing the brachial vein with a sterile 27G needle and transferring blood *via* heparinised capillary tubes (Hirschmann Laborgeräte GmBH, Germany) into Eppendorf tubes. Blood samples were kept on ice in an Eppendorf tube until we returned to the lab the same day, and then centrifuged for 10 min at 7000 rpm to separate plasma from red blood cells. Samples were stored in Eppendorf tubes at −50°C until subsequent laboratory analysis.

Immune Assays

We used two assays that focus on the innate immune system and one assay that focus on the acquired immune system (Hegemann et al., 2018a,b). The innate immune system constitutes the important first line of defence (Janeway et al., 2005). Levels of constitutive innate immune function relate to pathogen pressure (Horrocks et al., 2012, 2015) and show consistencies over longer time scales (Hegemann et al., 2012), but also experience modulations during migration (Eikenaar et al., 2020a,b). Specifically, we used a haemolysis-haemagglutination assay to quantify titres of complement-like lytic enzymes (measured as lysis titres) and non-specific natural antibodies (measured as natural antibody titres) and followed the method described by Matson et al. (2005). Scanned images of individual samples serially diluted in Rabbit red blood cells (Alsevers, S.BC-0009, Envigo, United Kingdom) were randomised among all plates and scored blindly to sample ID (by AH). If scores differed by >1, then a third score was taken and the median was used as a final value. We used a commercially available colorimetric assay kit (Tp801; Tridelta Development Ltd.) to quantify haptoglobin concentrations in plasma samples (Matson et al., 2012). We followed the manual method as described in the manufacturer manual with a few minor modifications. We measured absorbance at three wavelengths (405, 450, and 630 nm) prior to the addition of the final reagent that initiated the colour change reaction. The 405 and 450 nm pre-scan enabled us to statistically analyse and correct for differences in plasma sample redness, an indication of haemolysis, which can affect the assay (Matson et al., 2012; Wemer et al., 2021). Haptoglobin is an acute phase protein that circulates in the blood and during a pathogenic challenge haptoglobin concentration typically increases following release from the liver. Acquired immune function reflects the investment into immune function over longer time scales and hence is likely related to phenotypic quality (Hasselquist et al., 2001). We used an enzyme-linked immunosorbent assay (ELISA) to quantify the total level of antibodies (IgY) following the methods exactly as outlined in Sköld-Chiriac et al. (2014). Samples were defrosted and refrozen between each assay, but these assays are robust to repeated freeze-thaw cycles (Hegemann et al., 2017).

Molecular Analyses of Blood Parasites

DNA from preserved blood was extracted using standard phenol/chloroform methods (Sambrook et al., 2002) and subsequently diluted to 25 ng μl^{-1} (concentration determined using a nanodrop). To determine infection status with haemosporidian species of the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*, we used a nested PCR amplifying a partial segment of the cytochrome *b* gene and using both Haem-F/Haem-R2 and Haem-FL/Haem-R2L primer pairs following Hellgren et al. (2004). We subsequently grouped the data by birds having no infection, birds having one infection (*Haemoproteus*/*Plasmodium* or *Leucocytozoon*), or birds having double infection (*Haemoproteus*/*Plasmodium* and *Leucocytozoon*). Since we only consider first year birds in our analyses, all infections must have occurred on the breeding grounds and birds cannot yet be infected with lineages that are transmitted on the wintering grounds (see Hellgren et al., 2007).

Statistics

We analysed our data using linear mixed models [function lmer, package lme4 (Bates et al., 2015)] and the program R version 3.6.3 (R Development Core Team, 2020). Even though measurements of baseline immune function are usually not affected by short-term handling stress (Buehler et al., 2008a; but see Zylberberg, 2015), we first checked if handling time and immune parameters were correlated in our dataset, but this was not the case for any parameter (always $F < 2.31$, $p > 0.13$).

To separate within-species effects from between-species effects, we applied the methods described by van de Pol and Wright (2009). We used the immune parameters and blood parasite infection as dependent variable and the two-way-interactions of within-species-date effect and strategy (long-distance vs. short distance migrant), and between-species-date effect and strategy as explanatory variables. Species was included as random intercept effect. The model with haptoglobin included a measurement at 405 nm as covariate to correct for plasma redness as this measurement explained more variation than then 450 nm measurement (Matson et al., 2012; Wemer et al., 2021). Blood parasite infections were grouped into three levels (no infection, single infection, double infection).

We always started with the full model and removed interactions if $p > 0.1$ based on backward elimination and the drop1 function in R. We kept all fixed effects independent of significance. Assumptions of all models were checked on the residuals of the final model. Residuals from the model having agglutination as dependent variable deviated from normality and transformations did not improve the fit. However, since general linear models are robust against deviation from normality (Zuur et al., 2010), we still consider the results to be valid. We extracted and plotted marginal means using the function ggpredict [package ggeffects (Lüdecke, 2018)].

RESULTS

Overall, early migrating individuals of LDMs had lower lysis, higher immunoglobulin levels and a higher prevalence of blood parasite infections than late migrating individuals (**Figures 1**, **2** and **Table 1**). In contrast, among SDMs, early individuals did not differ from late individuals in lysis, but had lower immunoglobulin levels and lower prevalence of blood parasite infections than late migrating individuals (interaction within-species-date-effect × strategy: lysis: $p = 0.062$, immunoglobulins: $p = 0.048$, blood parasite infections: $p = 0.005$, **Figures 1**, **2**, for full statistical results, see **Table 1**). Agglutination and haptoglobin concentrations did not differ between early and late migrating individuals, and there was no effect of migration strategy (interaction within-species-date-effect × strategy: agglutination: $p = 0.283$, haptoglobin: $p = 0.713$; within-species-date effect: agglutination: $p = 0.404$, haptoglobin: $p = 0.984$, **Figures 1**, **2** and **Table 1**). In general, LDMs had lower haptoglobin concentrations ($p = 0.049$) and a non-significant trend for lower agglutination ($p = 0.087$) than SDMs (**Table 1**). Species-specific figures showing all individual data points can be found in the **Supplementary Figures 1–5**.

DISCUSSION

We show that several parameters of constitutive (baseline) immune function and blood parasite infections differ between early and late migrating individuals, and that these patterns differ between long- and short-distance migrants. Thus, our study suggests that over the autumn migration period immune function is modulated in different ways depending on migration strategy.

Specifically, early migrating LDMs had lower complement activity (lysis) than late migrating individuals, while there was no such difference in SDMs. That early and late individuals of LDMs differ indicates a trade-off with migration timing in these time-minimisers (Klaassen et al., 2012). Individuals migrating early may have to make a trade-off with investment in immune function, in this case complement activity, a parameter that has been linked to survival probabilities (Hegemann et al., 2015; Roast et al., 2020). While early departure from Scandinavia might be beneficial in terms of early crossing of the Sahara and hence early arrival at the wintering grounds (Alerstam and Hedenström, 1998) this may come at the cost of innate immune function which still develops in young birds after independence (Stambaugh et al., 2011; Vermeulen et al., 2017; van Dijk et al., 2020; Aastrup and Hegemann, 2021). The higher immunoglobulin levels in early migrating LDMs compared to late migrating individuals may seem to contradict this hypothesis. However, immunoglobulin levels are thought to reflect phenotypic quality/condition (Hasselquist et al., 2001) and particularly LDMs that will encounter more novel pathogens, may benefit from investing in this part of immune function to be prepared for entering (tropical) wintering quarters with a higher pathogen pressure (Westerdahl et al., 2014; O'Connor et al., 2020). Low immunoglobulin levels among late migrating LDMs may then suggest that these individuals are of lower quality/condition and that they can therefore not initiate migration as early as those with high immunoglobulin levels. For SDMs, immunoglobulin levels instead increased over the autumn migration period. This may reflect that among SDMs high quality/condition individuals are migrating later in the autumn. Alternatively, this might be an effect of that the levels of circulating immunoglobulins are increasing over the autumn in these young birds as they are exposed to more and more pathogens. Such an effect may be particularly visible in SDMs as those birds are on average 2 months older when they migrate as compared to LDMs, because SDMs hatch earlier and migrate later.

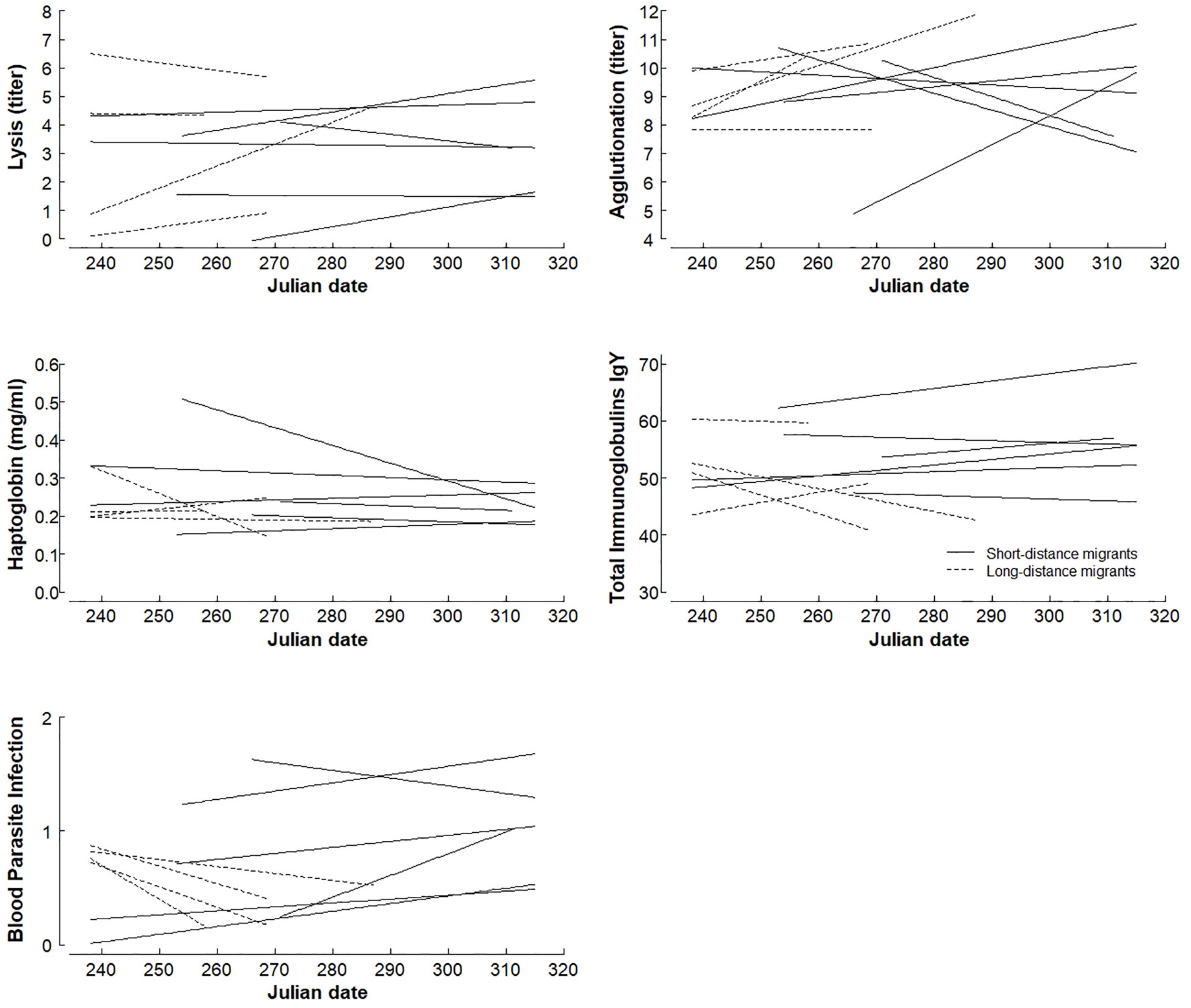

FIGURE 1 | Within-seasonal patterns of constitutive immune function and blood parasite infections in 417 individual migrants from 10 species sampled during autumn migration in Falsterbo, Sweden. Each line represents the relationship between Julian date and immune function or blood parasite infection for a single species, with dashed lines representing long-distance migrants (wintering in Africa) and solid lines presentencing short-distance migrants (wintering in Southern and Western Europe). Note that *y*-axis for agglutination and total immunoglobulins do not start at zero. Figures showing individual data points for each species can be found in the **Supplementary Material**.

Natural antibodies (agglutination) did not differ between early and late migrating individuals, which may suggest that this part of immune function is not involved in trade-offs related to migration. In line with this, several previous studies failed to find any correlation between agglutination and migration related traits (Buehler et al., 2010b; Nebel et al., 2012, 2013; Hegemann et al., 2018a,b; Eikenaar et al., 2020b). These studies as well as our current study instead suggest that other immune parameters (e.g., complement activity, haptoglobin concentration, immunoglobulins, bacteria killing capacity) seem to be more important for trade-offs between immune function and physiological or behavioural adaptations related to migration.

Haptoglobin levels did not differ between early and late migrating individuals. Since haptoglobin is an acute phase protein that increases during infection (Quaye, 2008), similar levels between early and late individuals might not be surprising. Interestingly though, LDMs had on average lower haptoglobin concentrations compared to SDMs. Besides its role for immune function, haptoglobin has antioxidant properties as it can bind haemoglobin, a mechanism potentially important during long endurance flights as haptoglobin levels were negatively correlated with flight duration in European Starlings (*Sturnus vulgaris*) flying in a wind tunnel (Nebel et al., 2012). Since LDMs tend to fly longer distances during each migratory flight compared

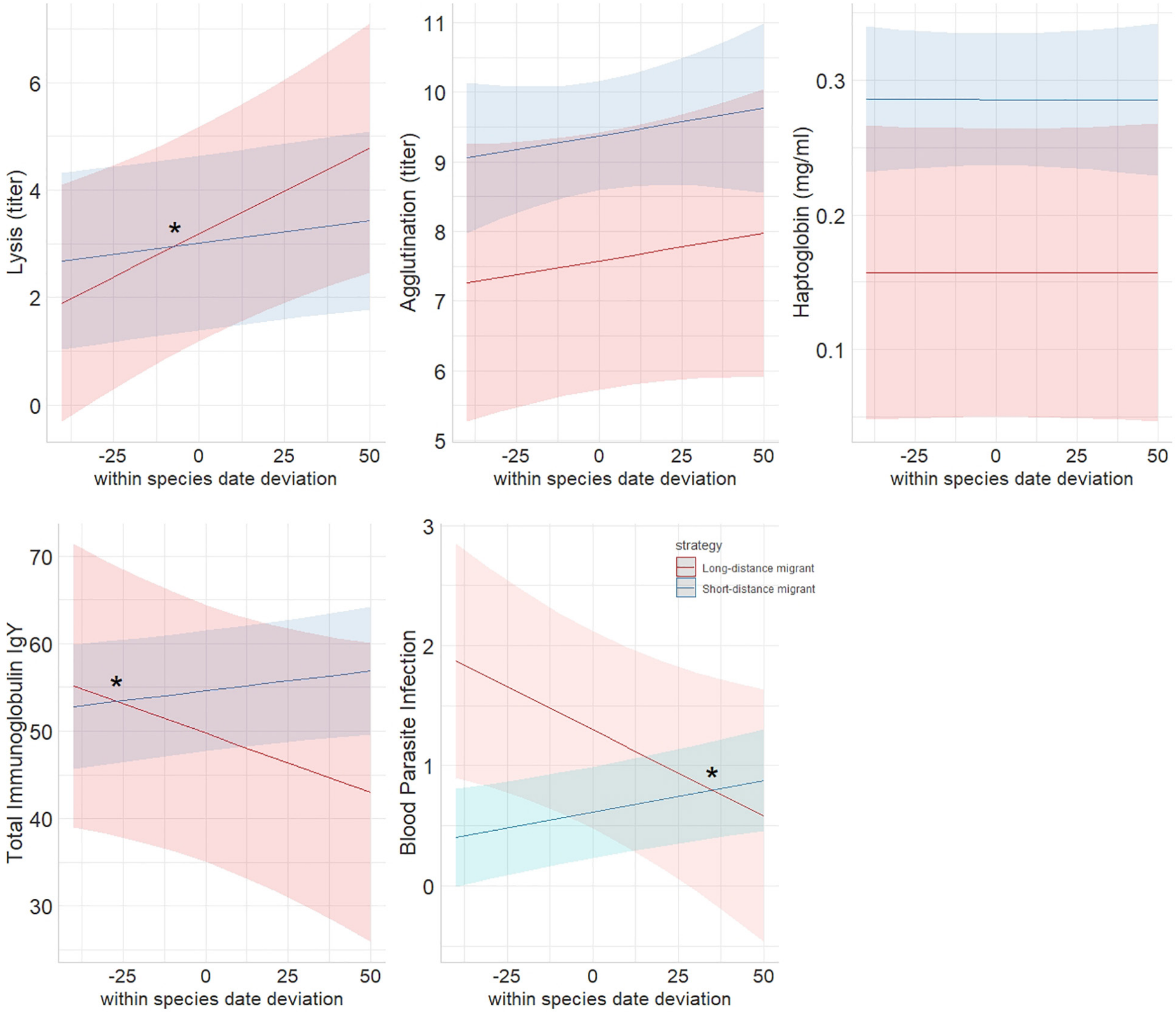

FIGURE 2 | Marginal means from linear mixed models showing within-species-date effect on immune function in long-distance and short-distance migrants sampled during autumn migration at Falsterbo, Sweden. A negative slope means that early migrating individuals have a higher value than late migrating individuals, a positive slope that late migrating individuals have higher values than early migrating individuals. Stars denote significant interactions. Haptoglobin significantly differed between the strategies. For details of statistical results, see section "Results" and **Table 1**.

TABLE 1 | Statistics and coefficients for linear mixed models analysing immune function and blood parasite infections in migrating birds during autumn migration at Falsterbo, Sweden.

	Lysis		**Agglutination**			**Haptoglobin**			**Immunoglobulins**		**Blood parasites prevalence**		
***X*-variable**	***F***	***p***	**Beta**	***F***	***p***	**Beta**	***F***	***p***	***F***	***p***	**Beta**	***F***	***p***
Strategy: between-species-date	5.17	**0.023**		2.19	0.139		0.25	0.620	1.84	0.175		0.13	0.717
Strategy: within-species-date	3.48	*0.062*		1.15	0.238		0.14	0.713	3.91	**0.048**		7.98	**0.005**
Between-species-date			−0.07	3.92	**0.048**		2.01	0.156	0.03	0.855	0.03	3.87	**0.049**
Within-species-date				0.70	0.404		0.00	0.984					
Strategy*			1.80	2.93	*0.087*	0.13	3.86	**0.049**					
Redness	NA	NA		NA	NA	0.59	384.3	**<0.001**	NA	NA		NA	NA

Within- and between-species effects were separated following the methods described by van de Pol and Wright (2009). Strategy: migration strategy (long-distance migrants vs. short-distance migrants). Redness: plasma redness to correct for plasma colour differences; relevant only for haptoglobin. p-Values $<$ 0.05 are bolded, p-values $<$ 0.1 $>$ 0.5 are italics.

**Short-distance migrant as reference.*

to SDMs (Schmaljohann, 2019; Packmor et al., 2020), this may explain the difference in haptoglobin between long- and short-distance migrants.

Prevalence of blood parasite infections increased in late migrating SDMs compared to early migrating birds and this is in line with findings in autumn migrating Robins in Hungary (Agh et al., 2019). An increase in blood parasite prevalence over the autumn in SDMs also supports the hypothesis that infected individuals migrate more slowly (Risely et al., 2018) and thus later, for example because of longer stopovers or shorter flight bouts (Hegemann et al., 2018b). Furthermore, infection with blood parasites can reduce feather growth (Marzal et al., 2013) and may delay the onset of migration in infected individuals. In contrast, migrating LDMs' prevalence of blood parasites decreased over the autumn migration period, which is contrary to our prediction and to previous results for LDMs [though during spring migration (Emmenegger et al., 2018)]. This contradicts the hypothesis of infected migrants being slowed down. However, since LDMs have the majority of their migratory flight still ahead of them, we cannot rule out that infected individuals migrate more slowly and hence are lagging behind uninfected individuals and that this pattern may only be visible later in the migratory season. Alternatively, but not mutually exclusive, early departure from the breeding grounds of infected individuals may be an adaptive strategy if they can anticipate that blood parasite infection may slow them down along the route. Another explanation may include selective disappearance of late migrating low-quality individuals infected with blood parasites (Marzal et al., 2016). Specifically, if late migrating long-distance migratory individuals are of poorer quality, as indicated by their lower levels of immunoglobulins (see above), those low-quality individuals may have reduced capacities to fight a haemosporidian infection (Gonzalez et al., 1999) and hence experience higher mortality during infection than early migrating high-quality individuals. This explanation may also resolve the apparent contradiction that early migrating LDMs have both high immunoglobulin levels (indicating high quality) and high prevalence of blood parasites. It seems unlikely that infections had already retracted into organs and were undetectable in the blood (Cosgrove et al., 2008), because prevalence increased over time and was on average much higher in late migrating SDMs. Furthermore, in a local bird community in southern Sweden, several long-distant migratory species showed no trend in parasite prevalence in late summer and early autumn when considering only first-year birds (Huang et al., 2020), and in blue tits *Plasmodium* blood parasites become dormant only from the end of October onwards (Cosgrove et al., 2008). Even though the overall patterns between blood parasite infections and immunoglobulins seem to parallel each other, we found no correlation on the individual level (Hegemann et al., in preparation).

CONCLUSION AND FUTURE DIRECTIONS

Our study adds to the growing literature that other physiological systems than those directly linked to energy turnover are modulated and thus variable during migration, and that this may be particularly true for the immune system (Buehler et al., 2010a; Hegemann et al., 2019) with potentially substantial impact on fitness-related factors (Roast et al., 2020; Dunn et al., 2021). That the different immune parameters did not show any common time patterns over the autumn migration period may highlight the different functions and costs of different immune parameters (Adamo, 2004; Matson et al., 2006; Demas et al., 2011) and that these trade-offs may differ between migration strategies. It also suggests that there is no general down-regulation or up-regulation of immune function, but that different parameters show different patterns within species, as well as between species using different migration strategies (i.e., short- and long-distance migrants). Hence, our results stress the importance of measuring multiple immune parameters in migration studies. The differences between early and late individuals also suggests that trade-offs between immune function and migration speed or other physiological systems might change over time as the migration season progresses. Future studies should aim at getting repeated samples from the same individuals both at the same stopover sites as well as during different stopovers along the migratory route to understand how flexible or fixed different immune strategies are. Satellite tracking of birds as well as radio-tracking in areas covered by a network of receiving stations like the Motus system (Taylor et al., 2017) may offer such possibilities. Understanding such variations in immune function will not only be vital for understanding the ecology and evolution of migration (Hegemann et al., 2019), but may also be important for predicting the interplay between birds and diseases under climate change (Hall, 2021).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors on request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Malmö/Lund Ethical Committee.

AUTHOR CONTRIBUTIONS

AH, J-ÅN, and DH: study design. AH: field work, statistical analyses, and first draft of the manuscript. AH and CB: labwork. AH, CB, DH, and J-ÅN: discussion and interpretation of data. All authors gave substantial input on the manuscript and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.880426/full#supplementary-material

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