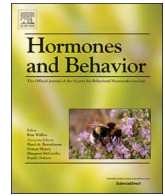




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Developing a Stopover-CORT hypothesis: Corticosterone predicts body composition and refueling rate in Gray Catbirds during migratory stopover

Joely G. DeSimone^{a,*}, Mariamar Gutierrez Ramirez^b, Cory R. Elowe^b, Michael S. Griego^b,
Creagh W. Breuner^{a,1}, Alexander R. Gerson^{b,c,1}

^a Organismal Biology, Ecology, and Evolution, University of Montana, Missoula, MT 59812, USA

^b Organismic and Evolutionary Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA

^c Department of Biology, University of Massachusetts Amherst, Amherst, MA 01003, USA

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ABSTRACT

Migratory flight is energetically challenging, requiring alternating phases of fuel catabolism and fuel accumulation, accompanied by dramatic changes in body composition and behavior. Baseline corticosterone (CORT; the primary glucocorticoid in birds) is thought to underlie transitions between fuel catabolism during flight, fuel deposition during stopover, and the initiation of migratory flight. However, studies of CORT on stopover physiology and behavior remain disparate efforts, lacking the cohesion of a general hypothesis. Here we develop a Stopover-CORT hypothesis formalizing the relationships among CORT, body condition, and refueling rate in migratory birds. First we expect body mass to increase with triglycerides (TRIG) as birds refuel. Second, based on a synthesis of previous literature, we predict a U-shaped CORT curve over the course of stopover, postulating that elevated CORT at arrival is reactive, responding to poor body condition, while CORT elevation before departure is preparative, driving changes in behavior and body condition. We tested these predictions in Gray Catbirds (*Dumetella carolinensis*) following a trans-Gulf flight during spring migration. We found baseline CORT was negatively correlated with body condition and TRIG, corresponding with our predictions for arriving and refueling—but not departing—birds. It is possible catbirds undergo regional habitat translocations rather than complete the entire stopover phase at our study site. We propose the Stopover-CORT hypothesis as a useful predictive framework for future studies of the mechanistic basis of stopover physiology. By studying the regulation of stopover refueling and departure, we may better understand physiological limitations to overall migration rate and improve assessments of habitat quality for refueling birds.

1. Introduction

Migration is a widespread behavior that has evolved repeatedly and independently in all major branches of the animal kingdom (Dingle and Drake, 2007; Bowlin et al., 2010). This behavior allows animals to take advantage of resources that vary in time and/or space (Alerstam et al., 2003; Dingle and Drake, 2007). For instance, many bird species migrate between wintering and breeding grounds each year, coordinating their movements with seasonal fluctuations in food availability. These migratory journeys are energetically expensive, requiring extensive physiological preparations for flight and recovery after flight at stopover sites along the way.

Both fat and lean mass undergo substantial, cyclical changes during migration: they increase prior to departure (Marsh, 1984; Jenni and

Jenni-Eiermann, 1998; Battley and Piersma, 2005; Guglielmo, 2018), are depleted during flight (Moore and Kerlinger, 1987; Piersma and Gill, 1998; Battley et al., 2000; Bauchinger and Biebach, 2001), and are replenished during stopover (Piersma and Jukema, 1990; Carpenter et al., 1993; Gannes, 2002), a cycle that continues until the bird reaches its final destination.

Due to significant in-flight reductions in fat stores and lean mass, stopover refueling is a crucial component of migratory journeys. In fact, a greater proportion of the migration period, and therefore energy, is spent at stopover sites than is spent in flight (Alerstam et al., 2003; Wikelski et al., 2003; Schmaljohann et al., 2012). Upon arrival at stopover sites, birds must replenish and accumulate fat and lean mass to prepare for the next leg of their journey. However, there are often delays in mass gain at stopover (Rappole and Warner, 1976; Karasov and

* Corresponding author.

E-mail address: joely.desimone@umontana.edu (J.G. DeSimone).

¹ Both authors contributed equally to this work.

Pinshow, 2000), likely imposed by functional losses due to organ catabolism during flight. Therefore, birds must first rebuild lean tissues, especially the digestive tract, before they are able to deposit fat (Carpenter et al., 1993; Karasov and Pinshow, 2000; Gannes, 2002). Importantly, the rate at which birds are able to recover after a long flight before departing on the next one can determine overall migration rate (Alerstam, 1991; Carpenter et al., 1993; Schmaljohann and Both, 2017).

In order to complete a successful migration, animals must be able to perceive and appropriately respond to environmental and endogenous cues to time their preparations and movements. Corticosterone (CORT), a pleiotropic steroid, is thought to regulate many of these behavioral and physiological responses to both external and internal cues during migration (Cornelius et al., 2013). CORT mediates organismal responses to environmental unpredictability, and supports the increased metabolic demands of life history stage transitions (Landys et al., 2006; Wada, 2008; Cornelius et al., 2013). CORT has been implicated in regulating locomotor activity (Breuner et al., 1998), foraging behavior (Dallman, 1993; Löhmus et al., 2006), and fuel use (Le Ninan et al., 1988; Dallman, 1993), and it has been shown to predict migratory departure from stopover sites (Eikenaar et al., 2017, 2018).

CORT can predict or promote changes in physiology and behavior at two scales: elevations in baseline levels of CORT often induce preparations for predictable life history stage transitions, including fledging or seasonal migration, while much higher stress-induced CORT levels rise in response to unpredictable perturbations, such as a harsh storm (Landys et al., 2006; Wada, 2008; Romero et al., 2009). Because seasonal migration is a predictable life history stage, it is likely not a stressful event: baseline CORT levels can predict and promote stopover physiology, and CORT may only reach stress-induced levels in birds with severely depleted energy stores (Gwinner et al., 1992; Falsone and Jenni-Eiermann, 2009).

This paper has two primary aims. First we synthesize the current state of information on CORT and body composition at stopovers to develop a testable hypothesis about how CORT may reflect or promote changes in body composition in refueling migratory birds at stopover. While previous studies have primarily focused on one phase of the stopover period (i.e. arrival, refueling, or departure from the stopover site), here we provide a framework of hypotheses about the role of CORT over the course of the entire stopover duration. Second, we test this Stopover-CORT hypothesis with a field study in Gray Catbirds (*Dumetella carolinensis*) captured at a spring stopover site on a barrier island off the Gulf coast of Florida. We propose this hypothesis as a useful predictive framework for future research on stopover physiology that may (1) reconcile previous equivocal studies, (2) uncover the hormonal mechanisms underlying extensive and rapid changes in behavior and physiology, (3) reveal limits on refueling rate (and therefore migration rate), and (4) improve assessments of habitat quality for migrating birds, based on their behavior and body condition at stopover sites. Understanding how CORT reflects physiology and behavior during the arrival, refueling, and departure phases of stopover allows for predictions of birds' experiences during the previous flight, and its decisions regarding appropriate timing of departure from the stopover site.

1.1. Hypothesis development

Here we review studies examining baseline CORT in migratory birds upon arrival at a stopover site, during refueling, and prior to departure. Within each section we will cover information regarding body condition and CORT physiology. We then use these studies to inform our hypothesis on the role CORT plays in regulating stopover metabolic ecology.

1.1.1. After arrival

1.1.1.1. Body condition. Spring migrants, especially those that cross

ecological barriers, often arrive at stopover sites in poor energetic condition, with decreased fat stores and lean mass. Relative to pre-flight individuals, fat stores can be significantly reduced in birds captured after flight (Moore and Kerlinger, 1987; Battley et al., 2000; Bauchinger and Biebach, 2001). For instance, Bar-tailed Godwits (*Limosa lapponica*) captured at a stopover site had lost an estimated 55% of their body mass during flight (Landys-Cianelli et al., 2002). Great Knots (*Calidris tenuirostris*) displayed an 85% reduction in fat mass, and about 20–30% reductions in pectoralis muscles, intestines, kidneys, and livers (Battley et al., 2000). However, headwinds or weather events may induce birds with sufficient fat stores to land, such that some arriving birds may not have depleted fuel stores (Moore and Kerlinger, 1987). Additionally, short-hop migrants may not deplete fuel stores and arrive in better condition (Schmaljohann and Eikenaar, 2017).

1.1.1.2. Corticosterone. Baseline CORT has repeatedly been shown to be negatively correlated with measures of body condition across taxa (Schwabl et al., 1991; Kitaysky et al., 1999; Moore et al., 2000; Romero and Wikelski, 2001), and this relationship has emerged in stopover physiology as well. In studies where birds were estimated to be captured within hours of arrival, CORT was negatively associated with body mass and fat stores (Jenni et al., 2000; Schwabl et al., 1991). However, Landys-Cianelli et al. (2002) did not observe a significant relationship between body condition and baseline CORT among Bar-tailed Godwits lured with playback to a false stopover site. Reneerkens et al. (2002) also failed to find a relationship between CORT and body condition in Bar-tailed Godwits that arrived at their breeding grounds but had not yet begun to breed. In this study, however, arrival time is imprecisely measured and includes birds that had been on the breeding grounds for up to 8 days. Importantly, these studies examine variation in baseline levels of CORT. Migratory flight is not stressful in and of itself: the baseline samples of CORT in migrating Garden Warblers (*Sylvia borin*) and European Robins (*Turdus migratorius*) only approached stress-induced CORT levels in individuals with severely depleted energy stores (Gwinner et al., 1992; Falsone and Jenni-Eiermann, 2009). Furthermore, captured migrants are able to mount a clear stress response, which is not possible if they are already in a stressed state (Schwabl et al., 1991; Romero et al., 1997; Landys-Cianelli et al., 2002).

1.1.1.3. Predictions for corticosterone and body condition. Given that birds often arrive in poor body condition and begin refueling on arrival, we predict that CORT levels will be elevated reflecting poor energetic condition, and decline as condition improves. In individuals in extremely poor energetic condition, CORT levels will be indicative of a mounted stress response, but in general we expect to observe only variation in baseline levels of CORT.

1.1.2. Refueling

1.1.2.1. Body condition. Birds often spend the stopover phase foraging, rebuilding tissues catabolized during flight, and depositing energy stores for the next leg of their journey. The body mass of Bar-tailed Godwits captured at a spring stopover site increased linearly over the entire stopover period (Piersma and Jukema, 1990). Blackcaps (*Sylvia atricapilla*) gain mass at stopover, but only after a 3–4 day period of low mass gain (Gannes, 2002). Carpenter et al. (1993) observed a pattern of bi-phasic mass gain characterized by an initial slow phase where lean mass was rebuilt followed by a rapid acceleration of body mass gain, where fat was deposited in refueling Rufous Hummingbirds (*Selasphorus rufus*). However, Kuenzi et al. (1991) observed mass loss over the course of stopover among several species of migratory passerines, which may be indicative of poor stopover habitat and food availability.

1.1.2.2. Corticosterone. Moderate elevations in CORT may promote foraging behavior (Landys et al., 2006). For example, baseline CORT undergoes daily cyclic rhythms, with peaks occurring before feeding

times in rats and humans, even after the regular feeding schedule is shifted (Dallman et al., 2004). Adrenalectomized rats display reduced daily food intake that can be restored via low doses of CORT (Dallman, 1993). When treated with RU486, which inhibits the low-affinity glucocorticoid receptor, captive Red Knots (*Calidris canutus islandica*) lowered the rate at which they visited their food dishes (Landys et al., 2004a) and spring migratory White-crowned Sparrows (*Zonotrichia leucophrys gambelii*) decreased food intake (Landys et al., 2004b). Compared to control birds, CORT-implanted captive White-crowned Sparrows showed prolonged foraging activity after a 3-day fast (Astheimer et al., 1992). The authors suggest CORT may help induce intense foraging behavior after periods of food deprivation, which may be relevant to migratory birds transitioning from extended flight to stopover. Additionally, captive Red-eyed Vireos (*Vireo olivaceus*) fed CORT-injected mealworms increased the rate at which they visited their food dishes (Löhms et al., 2006).

Other experimental manipulations of CORT, however, have not increased foraging behavior. For instance, captive Dark-eyed Juncos (*Junco hyemalis*) given CORT implants increased fat storage and atrophied flight muscles, but showed no difference in foraging behavior or food consumption (Gray et al., 1990). After experiencing a captive fasting-refeeding simulation of migratory stopover, Northern Wheatears (*Oenanthe oenanthe*) fed CORT-injected mealworms showed no difference in food intake compared to controls (Eikenaar, 2017).

It is difficult to know whether CORT manipulations appropriately simulate the relevant endogenous hormone levels experienced in the wild. Thus, some studies have taken a more correlative approach to examining the role of endogenous CORT levels on food intake. Eikenaar et al. (2013) found baseline CORT was negatively associated with fuel deposition rate in Northern Wheatears captured at a migratory stopover site. Similarly, Northern Wheatears experiencing a fasting-refeeding protocol displayed a negative relationship between baseline CORT and food intake while refueling (Eikenaar et al., 2014a, 2014b). Finally, in Red Knots held in captivity throughout the spring migratory period, baseline CORT was neither associated with increases in body mass in early spring nor with mass decreases at the end of the migratory phase (Piersma et al., 2000).

In the field, where food intake of individual animals is difficult to record, plasma triglycerides (TRIG) have been shown to accurately represent refueling rate (Guglielmo et al., 2005). As triglycerides are the storage form of lipids, an increase in circulating TRIG predicts body mass gain (Jenni-Eiermann and Jenni, 1994), and TRIG has been observed to increase over the course of stopover (Landys et al., 2005). Liu and Swanson (2014) observed a negative relationship between baseline CORT and TRIG and suggest that high quality habitats with sufficient resources for refueling may lower baseline CORT levels. In a study of partially migratory European Blackbirds (*Turdus merula*), Fudickar et al. (2013) observed greater fat scores and a trend towards higher baseline CORT levels in migrants compared to sedentary individuals, with no corresponding increase in TRIG.

The studies discussed here relating CORT to refueling behavior appear to have contradictory conclusions, with CORT being either positively or negatively related to foraging rate and behavior. One possible reason for these apparent contradictions is the feedback inherent in these relationships: variation in CORT levels can both cause and respond to foraging behavior. In birds in poor energetic condition, CORT may induce feeding behavior and fat deposition (positive relationship), but as these animals' body condition improves with increased fuel storage, CORT levels may decline in response (negative relationship).

1.1.2.3. Predictions for corticosterone, body condition, and refueling rate. In general, we expect body mass to increase over the course of stopover, with possible delays due to significant in-flight reductions in lean mass. TRIG is expected to increase as birds refuel during stopover, corresponding with increases in body mass. Elevated CORT levels early

in the stopover phase may promote refueling, due to both changes in behavior—increasing feeding rate—but also because CORT plays a role in energy mobilization and deposition and during anabolic processes may aid in the deposition of fat. CORT levels may then decline as birds deposit fat and increase body mass and condition.

1.1.3. Before departure

1.1.3.1. Body condition. As discussed above, birds typically increase body mass, fat mass, and lean mass during stopover. Birds are expected to depart their stopover site once they've acquired sufficient fuel stores for the next leg of their journey (Alerstam, 1991), though departure fuel load also depends on the site-specific fat deposition rate (Lindstrom and Alerstam, 1992). Thus departing birds are often in better body condition than arriving or refueling birds (Biebach et al., 1986; Moore and Kerlinger, 1987; Goymann et al., 2010).

1.1.3.2. Corticosterone. CORT is likely involved in the regulation of migratory departure because glucocorticoids are known to regulate locomotor activity (Breuner et al., 1998; Cash and Holberton, 1999) and elevations in baseline CORT support the increased metabolic demands of life history stage transitions (Landys et al., 2006; Wada, 2008). For instance, nestling baseline CORT levels begin to rise at least a few days before fledging (Heath, 1997; Schwabl, 1999; Sockman and Schwabl, 2001; Quillfeldt et al., 2007; Sprague and Breuner, 2010) and baseline CORT is elevated during periods of increased locomotor activity post-fledging (Belthoff and Dufty, 1998). These elevated levels are hypothesized to facilitate the increased locomotor and foraging activities of a bird outside its nest and/or changes in metabolism prior to fledging (Wada, 2008).

In an analogous fashion, CORT has been shown to increase in birds prior to migratory departure. Baseline CORT is positively associated with migratory restlessness (Löhms et al., 2003; Landys et al., 2004b), which is nocturnal hopping activity of captive birds, indicative of readiness for flight (Eikenaar et al., 2014b). Landys-Gianelli et al. (2002) observed baseline CORT levels increased with size-corrected body mass among Bar-tailed Godwits, possibly in preparation for migratory flight. Northern Wheatears with higher baseline CORT levels were more likely to depart on a given night, and among departing wheatears, those with higher CORT departed earlier in the night (Eikenaar et al., 2017). Finally, baseline CORT in captive Red Knots was highest when spring body mass reached maximum levels, presumably when they were ready for migratory departure (Piersma et al., 2000).

Increases in baseline CORT levels may “prime” the stress response axis (Sapolsky et al., 2000), such that pre-departure CORT elevations prepare birds for the unpredictable environmental, social, and metabolic challenges of long-distance flights and the breeding grounds (Piersma et al., 2000). Thus elevated CORT prior to predictable life history changes, including the onset of migration, may represent a physiological adjustment to support the increased metabolic demands of flight, and not a reactive response to adverse conditions (McEwen and Wingfield, 2003; Landys et al., 2006; Romero et al., 2009).

1.1.3.3. Predictions for corticosterone and body condition. We thus expect CORT and body condition to be positively associated in birds just prior to departure from a stopover site: these birds have regained body mass catabolized during flight and deposited energy stores for their next flight, and baseline CORT rises in preparation for migratory departure.

1.2. Hypothesis framework

Based on this review of the literature, we developed a hypothesis regarding the relationships among baseline CORT, body condition, and refueling rate of migratory birds at stopover. This Stopover-CORT hypothesis is depicted by Fig. 1.

We expect birds to improve their body condition and to refuel throughout the duration of stopover, increasing both their TRIG levels

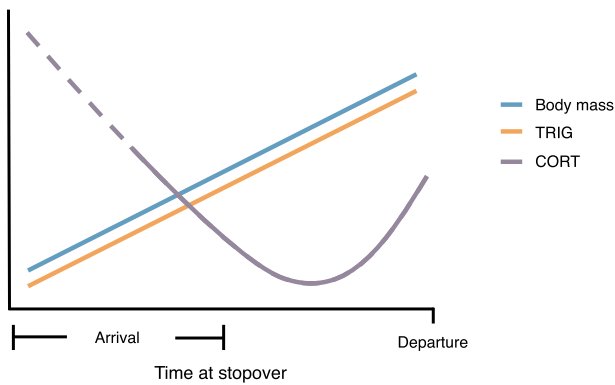


Fig. 1. Predicted relationships among body mass, plasma TRIG, and baseline CORT at stopover, from arrival at the stopover site on the left to departure from the site on the right. Birds arriving in better condition than others “arrive” further along the x-axis, showing the same progression of body condition, TRIG, and CORT, but beginning that process further along than birds arriving in poorer condition. CORT can reach stress-induced levels upon arrival at a stopover site in birds in very poor energetic condition, as indicated by the dashed part of the CORT curve; CORT levels of other individuals upon arrival may only represent variation in baseline levels (solid line). The elevation in CORT prior to departure is expected to represent a rise in baseline levels. Fat and lean mass are expected to follow the same trend as body mass.

and body condition over time. We expect baseline CORT to be elevated upon arrival—possibly but not necessarily to levels indicating a stress response—and to be negatively associated with body condition, such that birds arriving in poorer condition have higher baseline CORT levels. Additionally, we expect migrants that have completed long flights or crossed ecological barriers to show arrival physiology represented by the far left region of the x-axis in Fig. 1. Short-hop migrants should arrive in better condition (Schmaljohann and Eikenaar, 2017). Thus we expect the arrival physiology of birds arriving in better body condition to be represented by a region of the x-axis further to the right than that of birds arriving in poor condition (Fig. 1).

Elevated CORT may promote refueling in birds shortly after arrival, and baseline CORT is expected to decline as birds continue refueling and improve their energetic condition. Once birds have sufficiently refueled, we expect baseline CORT to increase prior to departure from stopover. This increase may begin a few days before departure, as it does in fledging birds (Sprague and Breuner, 2010), or just within an hour or two of departure (Eikenaar et al., 2020). Importantly, “departure” here means the onset of another migratory flight, not simply departure from the study site, because migrants often use a much broader stopover landscape before resuming migratory flight (Taylor et al., 2011).

For these reasons, we hypothesize inverse relationships between CORT and body condition upon arrival and prior to departure: CORT will be negatively associated with body condition among arriving and refueling birds and positively associated with body condition among birds ready to depart. These expected inverse relationships may be explained by different roles of CORT during different stopover phases. Among arriving birds, CORT levels are likely reactive to body condition: birds with low fuel stores are energetically challenged, which causes CORT elevations; among departing birds, CORT likely serves a preparatory role, inducing migratory behavior.

1.3. Case study

We conducted a case study to test the Stopover-CORT hypothesis by examining the relationships among CORT, body condition, and refueling rate in wild-caught songbirds during spring migratory stopover.

We captured Gray Catbirds (*Dumetella carolinensis*) on St. George Island, FL, a barrier island in the Gulf of Mexico. Billions of birds pass

through the Gulf region each spring on migration (Horton et al., 2019) and St. George Island can provide first landfall for trans-Gulf neotropical migrants (Lester et al., 2016), increasing the likelihood that captured birds are migratory and have just completed a long-distance flight. We analyzed the relationships among baseline CORT, body condition precisely measured using Quantitative Magnetic Resonance (QMR), and TRIG to examine the role of CORT in modulating migratory behavior.

A major inherent difficulty in studies of stopover ecology is producing accurate estimates of stopover duration (i.e. arrival and departure time) for individuals. To account for this issue, some studies have observed arrivals (Jenni et al., 2000), lured birds out of flight with playback or decoys (Landys-Cianelli et al., 2002), or flew trained, captive animals (Haase et al., 1986). Estimations of arrival time can be made based on known site- and species-specific temporal patterns of migratory flight (as in Schwabl et al., 1991; Paxton and Moore, 2017). Additionally, advances in radio telemetry have allowed for accurate recordings of departure time (as in Deppe et al., 2015; Eikenaar et al., 2017). Finally, analysis of stable hydrogen isotopes is a valuable tool to estimate the breeding latitudes and migration destinations of individuals in the absence of bands or transmitters (Hobson and Wassenaar, 1997). The catbirds captured on St. George Island may have completed a trans-gulf flight and used the island as a stopover site before heading to breeding grounds further north. However, St. George Island may not have been a stopover site for catbirds that over-wintered or planned to breed in the area. In this study we use knowledge of typical patterns of migrant body composition as well as feather stable-hydrogen (δD) isotope values to determine which of the Gray Catbirds we captured most likely used St. George Island as a stopover site.

2. Materials and methods

2.1. Study site

St. George Island is a barrier island approximately 8 km from the mainland coast of the Florida panhandle in the Gulf of Mexico (29.6678, -84.8656). This island often provides the first landfall for trans-Gulf neotropical migrants in spring (Lester et al., 2016). Forty-five km long, St. George Island is primarily residential, though the eastern 14 km are protected from development as the Dr. Julian G. Bruce St. George Island State Park. Fragmented lots along 2 km of the bayshore and adjacent to our field site are protected by the Apalachicola National Estuarine Research Reserve (ANERR). Vegetation includes slash pine (*Pinus elliotii*), live oak (*Quercus virginiana*), yaupon (*Ilex vomitoria*), and palmetto (*Sabal minor*).

2.2. Study species

Gray Catbirds winter throughout Mexico, Panama, and the Caribbean, and occur sparingly in northwest Colombia. Although Gray Catbirds also winter throughout southern Florida, the Central American migrants often use the barrier islands off the Florida coast as their first stopover after crossing the Gulf of Mexico. From there, catbirds continue on to breeding grounds in the southeast, northeast, and central regions of the United States, extending to parts of British Columbia and eastern Washington (Smith et al., 2011).

2.3. Capture and sampling

Gray Catbirds were captured using standard passive mist netting during spring 2017. From 01 April 2017 to 12 May 2017 as many as 16.5 standard size mist nets were opened between 0650–1300 and 1750–2000 daily. Each captured bird received a USGS aluminum leg band following standard banding protocols. We recorded morphometric measurements and body mass for all birds and age was determined according to Pyle (1997) when possible. In addition, each bird was

scanned in a Quantitative Magnetic Resonance (QMR) body composition analyzer, which accurately and non-invasively determines grams of fat and lean mass in a 90–120 s scan (Guglielmo et al., 2011). We collected one tail feather (R3) from each captured bird for stable isotope analysis of deuterium (δD). In all captured catbirds, we collected up to 200 μL of blood via brachial puncture with 27-gauge needles into heparinized capillary tubes. Blood samples taken within 3 min of capture ($n = 25$) were used to measure baseline CORT (Romero and Reed, 2005). Blood samples were kept on ice in the field and centrifuged later that same day. Plasma was stored in liquid nitrogen for the duration of the field season. Upon return to Massachusetts in May, plasma was stored in a $-80^\circ C$ freezer until TRIG analysis was completed and remaining plasma was shipped on dry ice to the University of Montana for CORT analysis. All procedures were conducted under the approval of UMass IACUC (#2015-0019) and state and federal permits (USFWS: MB65968B-0; USGS: 23979; Florida: LSSC-16-00033).

2.4. Corticosterone assay

Plasma corticosterone levels were detected using an Enzyme Linked Immunoassay (ELISA) kit (Cat No. 25-0412, Enzo Life Sciences). We conducted parallelism validation assays on a pool of 6 ether-extracted Gray Catbird samples to ensure accurate CORT measurements throughout the range of observed values. Standard dilution of plasma was parallel to the standard curve across dilutions 1:7–1:280. We chose 1:20 and 1:25 to balance plasma volume with placement in the standard curve (data not shown).

CORT was extracted from plasma with a double ether extraction after addition of 2000 cpm of 3H-CORT for recovery; ether was evaporated off in a $50^\circ C$ water bath, and sample was reconstituted in 250–400 mL assay buffer for a final dilution of 1:20 or 1:25. Recoveries averaged $78.2 \pm 2.0\%$; samples were corrected to 100% for analysis.

Assays were run as described in Wada et al. (2007). Briefly, 100 μL of extracted, diluted sample was assayed in triplicate if possible, and in duplicate when plasma volume was limiting. A standard curve (20,000 to 15.63 ng/100 μL) was included in triplicate on each of two plates, and an external CORT standard was also run in triplicate on each plate (100 μL /well). Color reaction was read at 405 nm corrected at 595 nm. Intra- and inter-plate coefficients of variance were 11.4 and 6.3% respectively. All samples were within the detectability limit for each assay.

2.5. Triglyceride assay

Glycerol and total triglyceride were determined spectrophotometrically using kits and a slightly modified protocol (Sigma Aldrich F6428; Sigma Aldrich T2449) as in Guglielmo et al. (2005). Plasma samples were diluted three-fold with 0.9% NaCl and triglyceride assays were run in duplicate in clear flat-bottom 96-well microplates and read at 540 nm in a microplate spectrophotometer (BioTek Synergy H, VT, USA). Concentrations were determined by comparison to a standard curve as per the kit instructions as in Guglielmo et al. (2005). Within-individual CVs were all $< 15\%$.

2.6. Stable isotopes

Feathers were stored at room temperature until processed. Feathers were washed of surface contaminants including lipids using 3–5 washes of chloroform:methanol 2:1, were allowed to air dry, and were stored in clean envelopes until analysis.

Approximately 0.2 mg of dried feather tissues was sealed in 3x5mm silver capsules and subjected along with reference materials to bench-top equilibration to local water vapor δD for at least three weeks prior to analysis to account for exchangeable hydrogen (Wassenaar and Hobson, 2000; Bowen et al., 2005). δD values of the non-exchangeable portion of hydrogen were determined by comparative equilibration

(Wassenaar and Hobson, 2003) using three internal laboratory keratin reference materials (δD : -55% , -95% , -175%) in which non-exchangeable δD values were measured by comparative equilibration experiments followed by external verification with other laboratories.

δD values were determined using a Thermo-Finnigan high-temperature conversion elemental analyzer (TCEA) coupled to a Thermo-Finnigan Delta Plus XL isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Isotopic results are expressed as δ values, $\delta D = 1000 * [(R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}}]$, where R_{sample} and R_{standard} are the $^2H/^1H$ of the sample and standard, respectively. The internationally accepted standard for hydrogen is Vienna Standard Mean Ocean Water (V-SMOW) and the units are expressed as parts per thousand, or per mil (‰). Precision for δD was determined by analysis of the three exchangeable (keratin) reference materials described above; within-run δD variation (SD) of these reference materials on the mass spectrometer system described above was $\leq 4\%$.

2.7. Body composition

We scanned birds in the QMR 2 to 3 times and averaged fat and lean mass across scans. Morphometric data from all 25 birds were used to create a scaled body mass index (as in Peig and Green, 2009). Tarsus was significantly associated with body mass ($F_{1,23} = 5.93$, $p = 0.023$) and lean mass ($F_{1,21} = 4.75$, $p = 0.04$), so tarsus length was used to scale body and lean mass. Fat mass was not significantly associated with any measured morphometrics and thus was not scaled by body size.

2.8. Migratory status

Populations of Gray Catbirds can winter and breed near our field site. To appropriately address our hypothesis, we focused our analyses on birds that most likely used St. George Island as a stopover site en route to breeding grounds further north. We employed two methods to filter our dataset accordingly.

First, we used body composition data to infer birds that were not in post-flight migratory condition, and therefore likely did not complete a trans-Gulf flight. To do so, we plotted the relationship between fat mass (g) and lean mass residuals, calculated from the regression of lean mass against tarsus (Fig. 2). This allowed us to visualize the overall condition of birds upon capture. We identified five individuals with negative lean

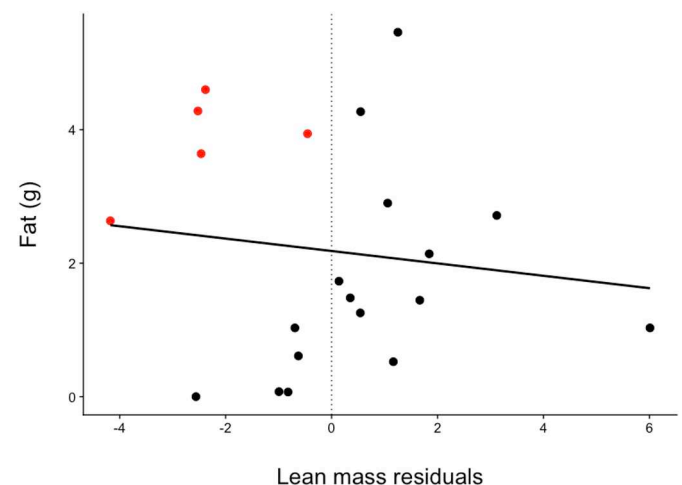


Fig. 2. The relationship between fat mass (g) and the residuals of lean mass (g) against tarsus length ($n = 21$). Five “atypical migrants,” shown in red, are identified with low lean mass residuals and substantial fat mass. These individuals were removed from the dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

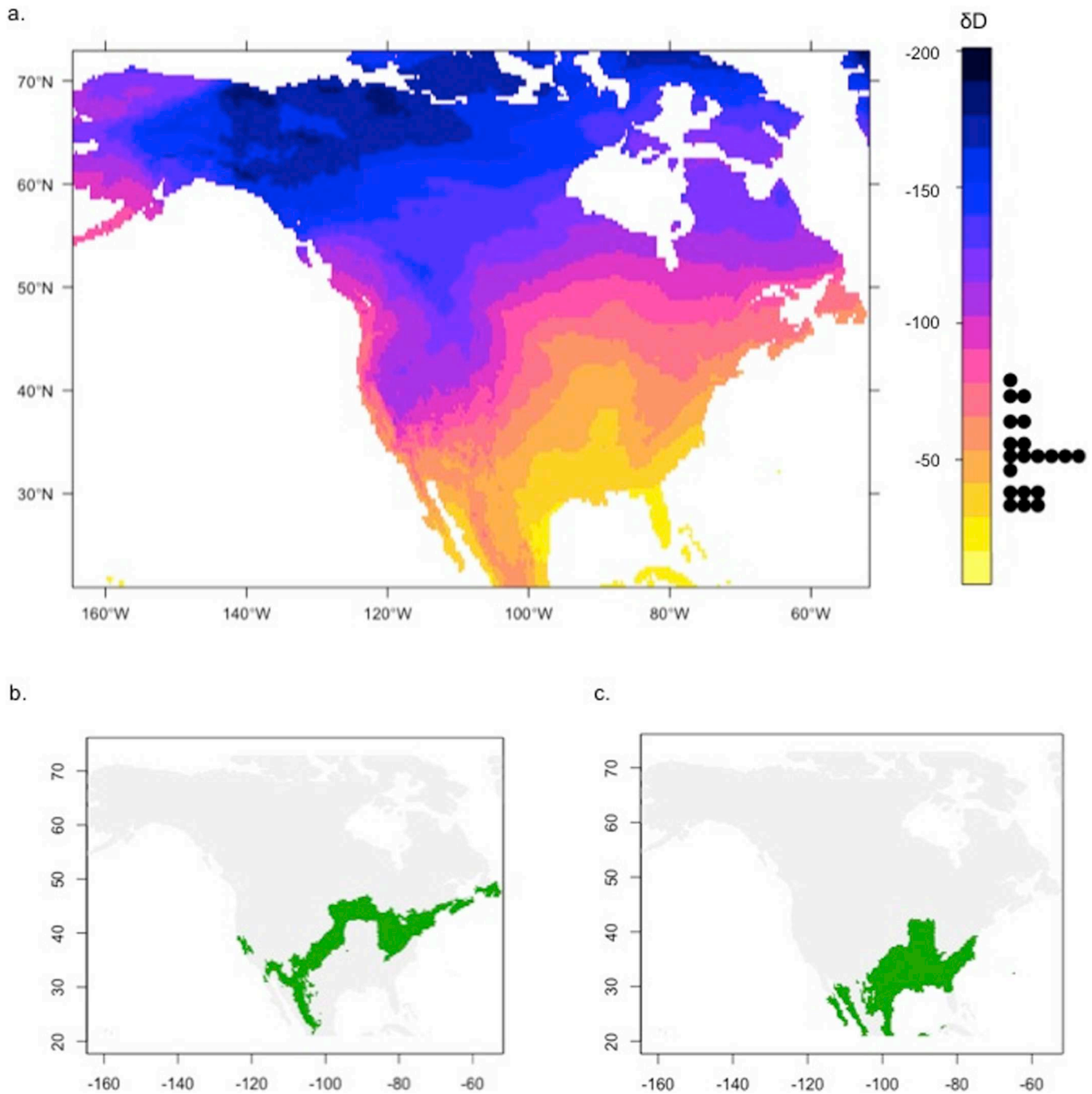


Fig. 3. a) Feather δD isoscape, whose legend includes a dotplot showing the δD values of each captured catbird. Below are examples of the top 10% of the posterior probability densities for two catbirds: the individual whose estimated breeding region is shown in (b) remained in our dataset, while we removed the individual shown in (c). The feather δD values for these individuals are -59.5 and -36.5 , respectively.

mass residuals but > 2.5 g of fat. These individuals are likely not trans-Gulf migrants because of their unusual body condition. Fat is the primary fuel for migratory flight (Jenni and Jenni-Eiermann, 1998), and although supplemental protein catabolism does occur during flight even while fat reserves are high (Gerson and Guglielmo, 2013), substantial protein catabolism is not expected until fat stores are low (Jenni et al., 2000). Furthermore, during stopover, lean mass is typically anabolized before fat deposition can occur (Carpenter et al., 1993; Karasov and Pinshow, 2000; Gannes, 2002). Thus, it is unlikely that long distance migratory birds at stopover would have relatively low lean mass while carrying substantial fat reserves. Therefore, these five individuals may have wintered on the island, close by, or on peninsular southern Florida

and are likely not trans-Gulf migrants.

Next, we used stable hydrogen isotopes to remove catbirds that would likely breed near our study site. We collected tail feathers, which were grown on or near the individual's breeding grounds the previous summer. δD values from these samples estimate the breeding latitude of individuals in the summer prior to being caught, and since the breeding site return rate of catbirds is about 25–30% (Darley et al., 1977; Ryder et al., 2011) birds will likely return to similar latitude. Using the assignR package (Ma and Bowen, 2019), we created a feather δD isoscape by first calibrating feather δD values of birds with known breeding locations to the environmental (precipitation) isoscape using data from Hobson et al. (2012). We then produced posterior probability densities

to estimate the breeding location of each individual. qtlRaster allowed us to visualize the 10% of grid cells with the highest posterior probability for each individual. We eliminated from our dataset samples ($n = 5$) whose top 10% probability region included our field site, which were also the samples with δD values > -45 (Fig. 3.) Wintering ground location cannot be similarly estimated for Gray Catbirds, because the isotopic signatures of southern Florida, the Caribbean, and northern South America are difficult to distinguish isotopically.

By applying these two analytical filters to our dataset, we increased the likelihood that our data reflect birds at a stopover site following a trans-Gulf flight and prior to further travel to the breeding grounds.

2.9. Statistical analysis

Samples were removed from the dataset if there was not corresponding QMR data ($n = 2$) or δD data ($n = 2$). One bird was removed because its CORT level was > 2 standard deviations above the mean. While all other samples were collected before 1300, this sample was collected after 1700. All statistical tests were performed using the mosaic package in RStudio (ver. 1.1.423) and data were visualized using the ggplot2 and gridExtra packages. We used generalized linear models with an alpha level of 0.05 to determine significance. The covariates initially included were Julian date of capture, time of day, age, and days since initial capture; only significant covariates remained in the final model, and these are noted with the results.

3. Results

The following results are from Gray Catbirds that used St. George Island as a stopover site ($n = 10$).

Eight CORT data points represent the first capture of an individual; two are from individuals captured 21 and 23 days after initial capture. We have repeated CORT measures for one individual, captured on days 0 and 21 of stopover. Of the 3 individuals with recaptures, none gained mass during their time at stopover (Fig. 4). On average, these birds lost 0.30 ± 0.14 g per day.

Baseline CORT reflected differences in body composition. The means, standard errors, and ranges of baseline CORT, plasma TRIG, and body composition are summarized in Table 1. CORT levels declined as scaled body mass increased ($p = 0.001$; $\beta = -1.31$; $R^2 = 0.83$; covariate: Julian date of capture; Fig. 5a). There was a non-significant

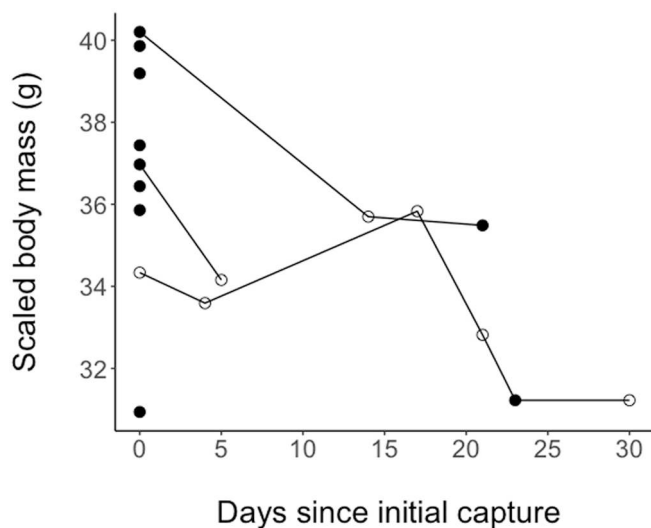


Fig. 4. Body mass at capture. Each point is an individual, and lines connect repeated capture events of the same individual. Solid circles represent captures for which we collected a baseline CORT sample; open circles don't have accompanying CORT data.

Table 1

Summary statistics of CORT, TRIG, and body composition ($n = 10$).

	Mean	SE	Range
CORT (ng/mL)	8.04	1.32	2.48–15.10
TRIG (mmol/L)	2.23	0.29	0.79–3.65
Body mass (g)	36.45	0.94	31.38–39.81
Fat mass (g)	1.67	0.43	0–4.27
Lean mass (g)	29.33	0.57	25.89–31.70

negative association between CORT and fat mass ($p = 0.08$; $\beta = -1.77$; $R^2 = 0.33$; covariates: none; Fig. 5b). CORT significantly decreased with increasing scaled lean mass ($p = 0.007$; $\beta = -1.46$; $R^2 = 0.80$; covariates: time of day, age; Fig. 5c) and plasma TRIG ($p = 0.051$; $\beta = -2.91$; $R^2 = 0.40$; covariates: none; Fig. 5d). These data are also visualized in Fig. S1 in the Supporting Information, which includes the individuals excluded from these analyses based on body composition and δD signature.

4. Discussion

Our study aims 1) to formalize the Stopover-CORT hypothesis regarding the relationships among body condition, TRIG, and CORT during migratory stopover and 2) to test this hypothesis in Gray Catbirds during spring migration. We found that baseline CORT predicts body condition. CORT levels decrease as scaled body mass, fat mass, and scaled lean mass increase. This result is consistent with the negative relationship between CORT and body mass seen in Bar-tailed Godwits (Ramenofsky et al., 1995; Landys-Cianelli et al., 2002) and several passerines (Jenni et al., 2000) at stopover. It has been proposed that baseline CORT is elevated during flight due to the metabolic demands of exercise (Ramenofsky et al., 1995; Piersma et al., 2000; Falsone and Jenni-Eiermann, 2009). It is important to note that catabolism and fuel type are regulated by variations in baseline CORT. Migratory flight is not inherently stressful, and CORT levels may only reach stress-induced levels when the bird is emaciated, with depleted fat stores and flight muscles (Gwinner et al., 1992; Jenni et al., 2000; Falsone and Jenni-Eiermann, 2009).

Contrary to our predictions, we did not observe a U-shaped curve of baseline CORT in relation to body condition. There are three explanations for this observation. First, our modest sample size, limited to morning and early afternoon captures, may make it difficult to observe the expected U-shape, especially if increases in CORT occur only in the hour or two before departure (Eikenaar et al., 2020). Second, Gray Catbirds may not complete their entire stopover period on St. George Island. The birds we captured may only represent the “arrival” and “refueling” phases of stopover, supported by the observed negative relationship between CORT and body condition. In this case, Gray Catbirds may relocate to a different part of the island or to the mainland in search of more suitable habitat to continue refueling before entering the “departure” phase and beginning the next leg of their northward journey. This idea is supported by automated telemetry data from other migratory species at our study site during the same year. The median length of stay on St. George Island of Swainson's Thrushes (*Catharus ustulatus*), Northern Waterthrushes (*Parkesia noveboracensis*) and Yellow-billed Cuckoos (*Coccyzus americanus*) in 2017 was 14.68 h, while the median stopover duration of these same birds within a stationary telemetry tower array up to 100 km north of the banding site was 107.5 h (M. Gutierrez Ramirez, unpublished data). Taylor et al. (2011) similarly observed such stopover relocations, rather than true departures, across diverse species and taxa during spring and fall migration. Furthermore, St. George Island is impacted by residential development, though the eastern end of the island, about 5 km from our banding site, is protected as the Dr. Julian G. Bruce St. George Island State Park. The mainland, about 8 km away, provides a greater area and diversity of refueling habitat.

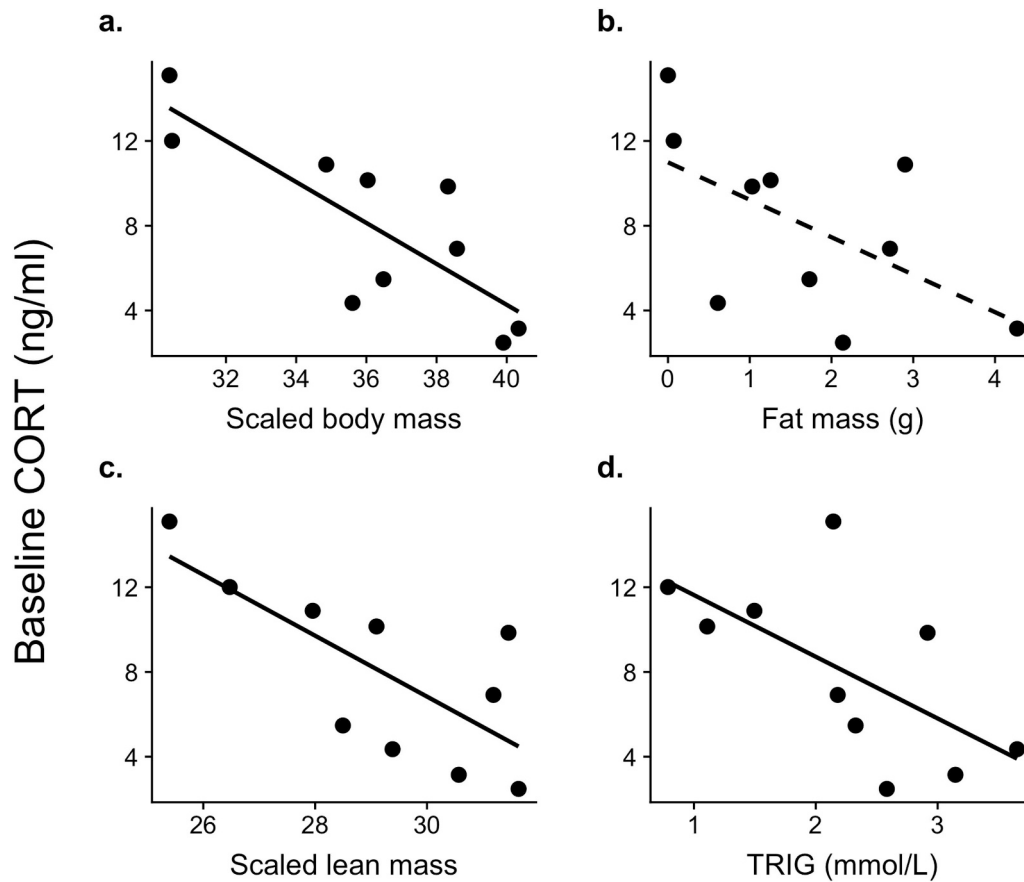


Fig. 5. Baseline CORT decreases as (a) scaled body mass, (b) fat mass, (c) scaled lean mass, and (d) plasma TRIG increase ($n = 10$). The dashed regression line in (b) represents a near significant trend.

Third, we may not have observed the predicted U-shaped curve because Gray Catbirds do not increase baseline CORT prior to departure. We think this is an unlikely possibility. During autumn migration, Holberton et al. (1996) found that catbirds in “migratory condition” (ie. post-molt and with sufficient fat stores) had higher baseline CORT than those in “pre-migratory condition” (ie. undergoing molt with little stored fat). Baseline CORT has been found to increase with fat scores or body mass among migratory European Blackbirds (Fudickar et al., 2013), Dark-eyed Juncos (Holberton et al., 2008), Bartailed Godwits (Landys-Cianelli et al., 2002), and Northern Wheatears (Eikenaar et al., 2017). In these studies, CORT was proposed to increase prior to departure to help regulate metabolism during flight and/or prepare individuals for unpredictable environmental conditions. It is possible that such an increase in CORT is unnecessary in catbirds completing short-hop flights over stopover areas on their way from St. George Island to their breeding grounds, but we do not know the nature of their flight patterns over the United States (Ryder et al., 2011).

We also observed a nearly significant negative relationship between CORT and refueling rate, as measured by plasma TRIG. The literature provides unclear data on the relationship between CORT and refueling. In this study, we show that CORT tends to be lower in birds with higher TRIG. This result is consistent with our predictions for birds in the earlier stages of stopover: as they refuel and improve their body condition, CORT levels decline.

Surprisingly, we found no mass gain between first and last capture of recaptured catbirds. Other studies have observed birds' failure to refuel at stopover sites because of physiological limitations due to protein catabolism during flight (Klaassen and Biebach, 1994), territoriality at the stopover site (Rappole and Warner, 1976), or insufficient habitat for refueling (Kuenzi et al., 1991). As noted above, it is possible

that St. George Island does not provide sufficient refueling habitat for migratory catbirds. However, the TRIG values of birds in this study are comparable to those of birds at other stopover sites (Guglielmo et al., 2005; Landys et al., 2005; Lyons et al., 2008).

It may be possible to estimate a bird's stopover phase (e.g. arriving, refueling, or departing) by examining the relationship between CORT and body condition and TRIG, in concordance with our hypothetical framework (Fig. 1). These results can be enhanced and potentially validated in future studies with the use of rapidly advancing tracking technology (McKinnon and Love, 2018). While arrival times are currently best approximated by observing arrivals (Jenni et al., 2000), luring birds out of flight (Landys-Cianelli et al., 2002), or capturing birds in flight (Jenni-Eiermann and Jenni, 1992; Falsone and Jenni-Eiermann, 2009), departure time can be precisely recorded using tracking devices (as in Deppe et al., 2015; Eikenaar et al., 2017). The advancement of efforts on minimization of radio tags will allow greater capacity to investigate stopover duration and habitat use in songbirds, augmenting the data presented here and further developing the Stopover-CORT hypothesis.

Given the difficulty of determining the time since arrival of migratory birds in most systems, and the seemingly contradictory relationships between CORT and body condition that we have reviewed in this manuscript, we believe that our proposed hypothesis provides a framework consistent with the available evidence, which will allow more accurate interpretation and insight into the role of CORT during stopover in migratory birds. Although our case study identified strong evidence in support of the relationship between body condition and CORT during the arrival and refueling period, the short stopovers typical of many songbirds on the barrier island system in the Gulf of Mexico, and the possibility that the island habitat quality is too low, or

competition among species too high, prevented a direct test of the relationship between CORT and departure on a migratory flight. Nonetheless, without regional automated telemetry, departures from the island would be interpreted as migratory departures, whereas in reality these were likely large-scale regional movements in search of better stopover habitat (Mills et al., 2011; Taylor et al., 2011), and this interpretation is supported by our proposed hypothesis.

In conclusion, we present the Stopover-CORT hypothesis as a predictive framework for future studies of stopover physiology and behavior. Examining the relationships between CORT, body condition, and TRIG may indicate which phase of stopover a bird is in. Deviations from the hypothesized pattern, as seen in our Gray Catbird case study, could allude to the stopover movement patterns of migratory birds and to the limitations of poor habitat quality or extensive competition that are worthy of future investigation.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yhbeh.2020.104776>.

Data availability

Data are available through the Dryad Digital Repository: <https://doi.org/10.5061/dryad.41ns1rmbb>.

CRedit authorship contribution statement

Joely G. DeSimone:Methodology, Conceptualization, Investigation, Formal analysis, Writing - original draft, Validation.**Mariamara Gutierrez Ramirez:**Investigation, Validation.**Cory R. Elowe:**Investigation, Validation.**Michael S. Griego:**Investigation, Validation.**Creagh W. Breuner:**Formal analysis, Writing - original draft, Validation.**Alexander R. Gerson:**Methodology, Conceptualization, Formal analysis, Writing - original draft, Validation.

Declaration of competing interest

The authors have no competing interests.

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