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Agusti Muñoz-Garcia

Sarah Aamidor

*See next page for additional authors*

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Muñoz-Garcia, A., Aamidor, S. E., McCue, M. D., McWilliams, S. R., & Pinshow, B. (2012). Allocation of endogenous and dietary protein in the reconstitution of the gastrointestinal tract in migratory blackcaps at stopover sites. *Journal of Experimental Biology*, 215, 1069-1075. doi: 10.1242/jeb.062547

Available at: <http://dx.doi.org/10.1242/jeb.062547>

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**Authors**

Agusti Muñoz-Garcia, Sarah Aamidor, Marshall D. McCue, Scott R. McWilliams, and Berry Pinshow

## RESEARCH ARTICLE

# Allocation of endogenous and dietary protein in the reconstitution of the gastrointestinal tract in migratory blackcaps at stopover sites

Agustí Muñoz-García<sup>1,\*</sup>, Sarah E. Aamidor<sup>1</sup>, Marshall D. McCue<sup>2</sup>, Scott R. McWilliams<sup>3</sup> and Berry Pinshow<sup>1</sup>

<sup>1</sup>Mitrani Department of Desert Ecology, The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Midreshet Ben-Gurion, Israel, <sup>2</sup>Department of Biological Sciences, St Mary's University, San Antonio Texas, TX 78228, USA and <sup>3</sup>Department of Natural Resources Science, University of Rhode Island, 1 Greenhouse Road, Kingston, RI 02881, USA

\*Author for correspondence (agustimg@gmail.com)

Accepted 22 November 2011

### SUMMARY

During migratory flight, the mass of the gastrointestinal tract (GIT) and its associated organs in small birds decreases in size by as much as 40%, compared with the preflight condition because of the catabolism of protein. At stopover sites, birds need 2–3 days to rebuild their GIT so that they can restore body mass and fat reserves to continue migration. The source of protein used to rebuild the GIT may be exogenous (from food ingested) or endogenous (reallocated from other organs) or both. Because the relative contribution of these sources to rebuild the GIT of migratory birds is not yet known, we mimicked in-flight fasting and then re-feeding in two groups of blackcaps (*Sylvia atricapilla*), a Palearctic migratory passerine. The birds were fed a diet containing either 3% or 20% protein to simulate different refueling scenarios. During re-feeding, birds received known doses of <sup>15</sup>N-L-leucine before we measured the isotope concentrations in GIT and associated digestive organs and in locomotory muscles. We then quantified the extent to which blackcaps rebuilt their GIT with endogenous and/or dietary protein while refeeding after a fast. Our results indicate that blackcaps fed the low-protein diet incorporated less exogenous nitrogen into their tissues than birds fed the 20% protein diet. They also allocated relatively more exogenous protein to the GIT than to pectoral muscle than those birds re-fed with the high-protein diet. However, this compensation was not sufficient for birds eating the low-protein diet to rebuild their intestine at the same rate as the birds re-fed the high-protein diet. We concluded that blackcaps must choose stopover sites at which they can maximize protein intake to minimize the time it takes to rebuild their GIT and, thus, resume migration as soon as possible.

Key words: blackcap, migration, stopover, gastrointestinal tract, stable isotope.

### INTRODUCTION

Birds that migrate long distances spend relatively short periods in flight that alternate with relatively long periods at stopover sites, where they replenish their energy stores and prepare for the next leg of migratory flight (McWilliams and Karasov, 2005; Wikelski et al., 2003; Bairlein and Gwinner, 1994; Biebach and Bauchinger, 2002; Weber, 2011; Piersma, 2011). The different nutritional and locomotory requirements during periods of flight and stopover demand dynamic structural and functional changes in some organs, mainly flight muscles and digestive organs. As a result, migrating birds have evolved a high degree of phenotypic flexibility in organ size and function (Battley et al., 2000; Karasov and Pinshow, 1998; Hume and Biebach, 1996; Schilch et al., 2002; Bauchinger et al., 2005; Piersma, 1998; Bauchinger and Biebach, 2001; Biebach and Bauchinger, 2002; McWilliams and Karasov, 2005). The study of phenotypic flexibility of the different organs of birds during migration gains evolutionary and ecological significance when one considers that reduction in size and, consequently, function of organs is associated with reduced energy expenditure (Biebach and Bauchinger, 2002; Battley et al., 2000; Piersma and van Gils, 2011), but, at the same time, imposes constraints on food processing rates (van Gils et al., 2003; van Gils et al., 2006), and thus the overall pace of migration (McWilliams and Karasov, 2005).

In preparation for migration, birds become hyperphagic and accumulate large quantities of body fat. Initially, it was thought that birds did not increase protein mass before migration (Odum et al., 1964); however, several studies have provided evidence to the contrary (Barlein and Gwinner, 1994; Fry et al., 1972; Lindstrom and Piersma, 1993; Piersma et al., 1999; Karasov and Pinshow, 1998). Most birds do not eat or drink in flight and thus must rely on their fat and protein stores as sources of energy and water (Bairlein and Gwinner, 1994; Karasov and Pinshow, 1998; Jenni and Jenni-Eiermann, 1998; Battley et al., 2000; Bordel and Haase, 2000). Because birds catabolize gastrointestinal tract (GIT) protein during migratory flight, the size of the GIT decreases by as much as 40% (Biebach, 1998; Hume and Biebach, 1996) and it follows that the bird's capacity to process food is also reduced by approximately the same fraction (Piersma et al., 1993; van Gils et al., 2006).

Phenotypic flexibility of key organs might benefit migrating birds in flight for several reasons. Locomotory costs are reduced because of the decrease in body mass ( $m_b$ ) resulting from fat catabolism and the decrease in GIT mass. The consequent decrease in mass permits a reduction in flight muscle size, reducing energy consumption and migration time (Bauchinger and Biebach, 2005; Lindstrom and Alerstam, 1992; Hendstrom and Alerstam, 1998). Also, the

catabolism of protein not only provides energy, but also supplies significantly more water per unit dry mass catabolized than fat (Klaassen, 1996; Jenni and Jenni-Eiermann, 1998; Bauchinger and Biebach, 1998; Gerson and Guglielmo, 2011). Bauchinger and McWilliams (Bauchinger and McWilliams, 2010) hypothesized that the different rates of mass loss of organs stems from the fact that each tissue has a different rate of protein turnover. Because birds do not ingest protein during flight, those tissues with high turnover rates will be the first to degrade. Consistent with this hypothesis, digestive organs have the highest protein turnover rates in birds and they are among the most phenotypically flexible organs (Starck, 1999; Bauchinger and McWilliams, 2010; Piersma and Van Gils, 2011).

During stopovers, the processes of ingestion, digestion and absorption of nutrients should operate at full capacity in preparation for the next leg of the bird's migratory flight, and thus migrating birds must fully restore the structure and function of the GIT at each stopover site (Karasov and Pinshow, 1998; Karasov and Pinshow, 2000; Piersma et al., 1999; Bauchinger et al., 2005). As the mass and, ultimately, the function of the GIT are reduced during flight, migrating birds stopping to refill energy stores face the problem of digesting food with a reduced digestive tract. Birds appear to, at least in part, mitigate this challenge behaviorally and physiologically by, for example, increasing food intake rates and selecting sites where high quality food may be found, and reducing gut transit time (Battley et al., 2005; van Gils et al., 2005; Bauchinger et al., 2009; Karasov and Pinshow, 2000). However, passerine migrants require some 24–48 h to fully recover the mass and function of the GIT, despite the abundance of food often encountered at stopover sites (Hume and Biebach, 1996; Karasov and Pinshow, 2000; McWilliams and Karasov, 2001; Klaassen and Biebach, 1994; Gannes, 2002; Aamidor et al., 2011). Although ecological challenges, such as interspecific and intraspecific competition, and handling time of new food items, have been invoked to explain the above phenomenon (Rappole and Warner, 1976; Moore and Yong, 1991; Starck, 1999; Schilch and Jenni, 2001; McWilliams and Karasov, 2005), physiological factors are apparently more important in accounting for the delayed rebuilding of the GIT in migrating birds at stopover sites (Karasov and Pinshow, 1998; Karasov and Pinshow, 2000; McWilliams and Karasov, 2001; Pierce and McWilliams, 2004; Aamidor, 2011). Birds at stopover sites cannot immediately feed at full capacity and need some time to restore their protein reserves, and GIT, before they begin to fatten (McWilliams and Karasov, 2001; Gannes, 2002; Aamidor et al., 2011).

Sources of protein are either recently ingested food or endogenous reserves (Bauchinger and Biebach, 2001). The most likely source of endogenous protein is flight muscle. To fly, flight muscles must be fully functional, but during a stopover they need not be so, and their protein might be allocated to elsewhere in the body (Bauchinger and Biebach, 2001; Biebach and Bauchinger, 2002). However, the proportion of exogenous and endogenous protein, and the origin of endogenous protein, that migrating birds use to restore their GIT at stopover sites is not known.

We investigated the sources of the protein used by migrating blackcaps (*Sylvia atricapilla*) during the early stages of rebuilding the GIT after a period of simulated in-flight starvation. The first 2 days at stopover sites represent a crucial period for birds to regain full structure and function of their GIT, which will allow them to rapidly refuel and resume migratory flight. We hypothesized that the availability of dietary protein at stopover sites influences the allocation of exogenous and endogenous protein used by migrating

birds to rebuild their GIT. We predicted that birds feeding on low-protein diets at stopovers rebuild their GIT more slowly, and use a higher proportion of endogenous than exogenous protein to do so, than birds eating high-protein diets. To test these predictions, we simulated in-flight starvation in captive blackcaps and then re-fed them for 2 days with either a low-protein (3%) or a high-protein (20%) diet. During the re-feeding period, all birds were administered a dose of stable-isotope-labeled essential amino acid  $^{15}\text{N}$ -L-leucine that allowed us to estimate the relative amount of exogenous protein used to rebuild certain organs in relation to diet quality and recovery from fasting.

## MATERIALS AND METHODS

### Capture of birds and experimental design

We mist-netted blackcaps [*Sylvia atricapilla* (Linnaeus 1758)] in Midreshet Ben-Gurion, Israel (30°51'17"N, 34°46'59"E) between March and May 2009, during spring migration, when the birds fly from their wintering grounds in Africa to their breeding grounds in Europe. Immediately after capture, we weighed the birds ( $\pm 0.01$  g) and measured the length of the tibiotarsus, bill, head-plus-bill and right wing, using Vernier calipers ( $\pm 0.1$  mm). Sex was determined by plumage characteristics – females have a rufous cap, males a black one. The blackcaps ( $N=5$  males and 14 females) were held in individual cages in an outdoor aviary for approximately 1 week. They were provided with a mixture (by mass) of 25% commercial dry insect-based food (Fettmischung fein Aleckwa, Tiernahrung, Altrip, Germany), 30% minced hard-boiled eggs, 21% curd, 15% bread crumbs, 6% ground egg shells, 3% minced beef heart and 0.53% vitamins (Vitakalk and moult vitamins, Södra Vallgrund, Finland) (Gwinner et al., 1988), supplemented with 20–50 mealworms per day per bird. Thereafter, the birds were transferred to a temperature-controlled room kept at  $35\pm 0.2^\circ\text{C}$  during the day and  $15\pm 0.2^\circ\text{C}$  at night, with constant vapor density, and a light regime following the natural photoperiod ( $\sim 15\text{h}:9\text{h}$  light:dark). In the temperature-controlled room, birds were transferred to individual cages and fed the above-described diet until they reached an  $m_b \geq 17$  g, the mass of free-living blackcaps caught during spring migration at Midreshet Ben-Gurion [ $17.3\pm 0.5$  g (Karasov and Pinshow, 1998)]. The day this occurred for each bird was considered day 1 of the experiment for that animal.

During the morning of day 1, we took a blood sample from the humeral vein and a biopsy of the pectoral muscle with a biopsy needle (Wescott 20 G  $\times$  3.5", Cardinal Health, Dublin, OH, USA). We used these samples to determine baseline values for the nitrogen isotope ratios of the tissues of birds. Beginning on day 2 of the experiment, birds were deprived of food and water for 2 days or until their  $m_b$  reached a lower limit of 12 g. After food deprivation, on day 4, the blackcaps were randomly assigned to one of two isocaloric diet groups: a high-protein diet consisting of 20% protein, 12% fat and 68% carbohydrates, and five mealworms per day ( $N=9$  birds), or a low-protein diet, similar to the former, but with 3% protein, and carbohydrates making up the necessary caloric difference ( $N=10$  birds; Table 1). We measured the food intake of each bird on both days of re-feeding. Throughout the experiment, at sunrise each day, we weighed all the birds to  $\pm 0.1$  g. On days 2, 4 and 6 we determined the proportion of lean mass and fat mass of the birds by dual energy X-ray absorptiometry (DEXA) using a Lunar PIXImus<sup>TM</sup> 2 (General Electric Medical, Fitchburg, WI, USA), following Korine et al. (Korine et al., 2004), who validated its use with small birds. In addition, we measured resting metabolic rate (RMR) by indirect calorimetry on the first day of food deprivation (day 2), the first day of re-feeding (day 4), and the last

Table 1. Composition of the two semi-synthetic diets fed to blackcap warblers

Ingredient	Mass (g)	
	High protein diet (55% carbohydrate, 20% protein, 10% fat)	Low protein diet (71% carbohydrate, 3% protein, 10% fat)
D-Glucose <sup>a</sup>	45.8	29.5
Fructose <sup>a</sup>	25.0	25.0
Casein <sup>b</sup>	2.4	16.8
Cellulose <sup>c</sup>	2.9	2.7
Salts <sup>d</sup>	6.7	6.7
Olive oil	10.0	10.0
Amino acid mixture <sup>e</sup>	0.7	2.8
Vitamins <sup>f</sup>	1.5	1.5
Agar <sup>g</sup>	5.0	5.0
Total dry	100	100
Water (ml)	300	300
Total wet	400	400

These ingredients were mixed with 300 g water per 100 g dry mix and offered to the birds as a wet mash. See Afik et al. (Afik et al., 1997) and Podlesak and McWilliams (Podlesak and McWilliams, 2006) for the recipe.

<sup>a</sup>Dextrose (Corn Products International, Westchester, IL, USA); fructose (Galum, Kibbutz Maanit, Israel).

<sup>b</sup>Casein (high N) (US Biochemical Corporation, Cleveland, OH, USA).

<sup>c</sup> $\alpha$ -Cellulose (Sigma-Aldrich, St Louis, MO, USA).

<sup>d</sup>Briggs-N Salt mixture (ICN Biomedicals, Solon, OH, USA).

<sup>e</sup>Mixture from Murphy and King (Murphy and King, 1982) (Fisher Scientific, Pittsburg, PA, USA).

<sup>f</sup>AIN-76 Vitamin and Mineral Mix (ICN Biomedicals, Inc., Irvine, CA, USA).

<sup>g</sup>Bacteriological grade (US Biochemical Corporation).

day of re-feeding (day 6), at sunrise, using an open flow respirometry system (described below). On the last day of the experiment (day 6), we killed the birds by decapitation and dissected out the organs of interest (see below). Intestines and gizzards were opened and rinsed in an isotonic saline solution to remove any undigested food. We also determined the wet mass of organs, and the dry mass after desiccating them to constant mass in an oven at 60°C for 48 h.

#### Measurement of resting metabolic rate of blackcaps

We measured oxygen consumption ( $\dot{V}_{O_2}$ ) of birds three times, always beginning at sunrise, using standard flow-through respirometry methods: at the beginning of the experiment, after food deprivation and after re-feeding, stages 1, 2 and 3, respectively. Postabsorptive birds were placed in 1.91 metabolic chambers, made from commercial hermetically sealable containers (Lock&Lock HPL9314, Hana Cobi, Korea), in a temperature-controlled cabinet (Thermo Scientific, model Precision 815, Cleveland, OH, USA), wherein air temperature was kept constant at 30.0±0.5°C, which is within the thermoneutral zone of blackcaps (Kendeigh et al., 1977). The  $\dot{V}_{O_2}$  of two to five animals was measured simultaneously on any given day. Air, purged of CO<sub>2</sub> and dried with a purged gas generator (Pure Gas, model PCDA-1-12-m-32-C, Broomfield, CO, USA), was routed through a manifold that distributed it to the metabolic chambers. Chamber-inlet airflow rates were controlled by an eight-channel flow metering system (Flow-Bar 8, Sable Systems, Las Vegas, NV, USA) set at 800–850 ml min<sup>-1</sup>. Air exiting the chambers passed through a dew point meter (RH-100, Sable Systems), and then through a column of magnesium perchlorate to remove water vapor. A subsample of the air was directed to a CO<sub>2</sub> analyzer (CD-3A, Applied Electrochemistry, Sunnyvale, CA, USA), and then to an oxygen analyzer (S3 A-II, Applied Electrochemistry) at a flow rate of ~100 ml min<sup>-1</sup>.

After 2 h of equilibration, we continuously recorded the fraction of O<sub>2</sub> and CO<sub>2</sub> ( $F_{O_2}$  and  $F_{CO_2}$ , respectively) in the excurrent air with a data logger (Campbell Scientific 21X, Logan, UT, USA), dew-point temperature with a water vapor analyzer (RH 300, Sable Systems) and the temperature in the chamber with a type-T thermocouple. We calculated  $\dot{V}_{O_2}$  using eqn 4 of Hill [(Hill, 1972), see p. 262 in Withers (Withers, 1977)] and converted it to mW using 20.08 J ml<sup>-1</sup> O<sub>2</sub> (Schmidt-Nielsen, 1997). We recorded  $m_b$  and body temperature ( $T_b$ ) of the birds before and after metabolic rate (MR) measurements.

#### Stable isotopes

On day 1 of the experiment, we took a blood sample and a biopsy of pectoral muscle for isotope analysis. On days 4 and 5, we gavaged the blackcaps with 200 µl of an aqueous solution of <sup>15</sup>N-L-leucine, 99% (Cambridge Isotope Laboratories, Andover, MA, USA) at a concentration of 13.5 mg ml<sup>-1</sup>. On the morning of day 6, birds were killed by decapitation. We collected approximately 1 mg of each of the following tissues for isotope analyses: liver, intestine, gizzard, blood, pectoral muscle and leg muscles (fibularis longus, gastrocnemius, tibialis and extensor). All tissues collected during the experiment were immediately frozen and then freeze-dried for 48 h and stored at -20°C. Samples were sent to the US Environmental Protection Agency Atlantic Ecology Division laboratory (Narragansett, RI) for isotope analyses.

Homogenized tissue samples were weighed to ±0.0001 g on a semi-microbalance (Precisa 40SM-200A, Precisa Gravimetrics AG, Dietikon, Switzerland) and put in tin cups (Costech, Valencia, CA, USA). Homogenates were placed in oxidation/reduction furnaces, and the evolved gas was separated by gas chromatography. We measured N isotope ratios using a Carlo-Erba NA 1500 Series II Elemental Analyzer (Lakewood, NJ, USA) interfaced with an Elementar Optima™ isotope ratio mass spectrometer (Beverly, MA, USA), with powdered dogfish muscle (DORM-1, National Research Council, Institute for Environmental Chemistry, Ottawa, ON, Canada) as a reference standard. N stable-isotope ratios are expressed in  $\delta$  notation as parts per thousand (‰) and compared to atmospheric air.

To evaluate the preferential routing of labeled leucine to each tissue in blackcaps, we compared the isotope ratios of each tissue with the isotope ratio of total body N, assuming the tracer was homogeneously distributed in the body after administration (McCue, 2011; McCue et al., 2011a). Making the assumption of homogeneous distribution is prudent and conservative because there are no published estimates of N turnover rates for organs of blackcaps or other small songbirds. Total <sup>15</sup>N content (<sup>15</sup>N<sub>total</sub>) in birds was calculated according to the following equation, and expressed in units of atom percent (atom%):

$$^{15}\text{N}_{\text{total}} = ^{15}\text{N}_{\text{background}} + ^{15}\text{N}_{\text{enrichment}} \quad (1)$$

<sup>15</sup>N<sub>background</sub> (atom%) was calculated from the average  $\delta^{15}\text{N}$  of blood and pectoral muscle of the experimental birds before they were administered with labeled leucine, according to Slater et al. (Slater et al., 2001):

$$^{15}\text{N}_{\text{background}} = 100 / (\{1/[R(\delta^{15}\text{N}/1000) + 1]\} + 1), \quad (2)$$

where  $R$  is the ratio of heavy to light nitrogen in atmospheric air (0.003663033).

<sup>15</sup>N<sub>enrichment</sub> (atom%) was calculated for each tracer dose according to Eqn 3:

$$^{15}\text{N}_{\text{enrichment}} = (^{15}\text{N}_{\text{exogenous}} \times 100) / (\text{N}_{\text{exchangeable}} + ^{15}\text{N}_{\text{exogenous}}). \quad (3)$$

We defined  $^{15}\text{N}_{\text{exogenous}}$  as the pool of administered, assimilated and retained  $^{15}\text{N}$  atoms, calculated as:

$$^{15}\text{N}_{\text{exogenous}} = m_{\text{dose}} F_{15\text{N dose}} F_{\text{assimilated}} (1 - F_{\text{oxidized}}), \quad (4)$$

where  $m_{\text{dose}}$  is the mass of the tracer dose in milligrams,  $F_{15\text{N dose}}$  is the mass fraction of  $^{15}\text{N}$  in each tracer molecule (i.e.  $15/131.17=0.114$ ),  $F_{\text{assimilated}}$  is the fraction of assimilated tracer, estimated to be 0.90 (Bairlein and Simons, 1995; Caviedes-Vidal and Karasov, 1996; Chediack et al., 2001; Chung and Baker, 1992; Hurwitz et al., 1973; Renner and Hill, 1961), and  $F_{\text{oxidized}}$  is the fraction of the tracer dose that was oxidized prior to death, as determined by breath testing, and estimated to be 0.143 for leucine (McCue et al., 2010; McCue et al., 2011b).

$\text{N}_{\text{exchangeable}}$  is the total pool of exchangeable nitrogen, and was calculated as:

$$\text{N}_{\text{exchangeable}} = m_{\text{dry}} F_{\text{bodyN}}, \quad (5)$$

where  $m_{\text{dry}}$  is the dry lean mass of the bird in milligrams, calculated as total dry mass minus fat mass, determined by DEXA for each bird, and  $F_{\text{bodyN}}$  is the mass fraction of nitrogen in the body, estimated to be 0.15 (Chibnall et al., 1943).

$^{15}\text{N}_{\text{total}}$  was then back-calculated to  $\delta^{15}\text{N}$ , which is the isotope ratio of  $^{15}\text{N}$  in the body following administration, assuming that the isotope is homogeneously distributed in the body. We compared these values with direct measurements of the  $\delta^{15}\text{N}$  of tissues after dose administration to determine whether  $^{15}\text{N}$  was preferentially routed to specific tissues and accepted that preferential allocation of nutrients had taken place when  $^{15}\text{N}$  enrichment of a particular tissue was greater than the expected  $^{15}\text{N}$  enrichment (McCue, 2011).

Because  $^{15}\text{N}$  of each tissue correlates with dry mass of the tissue, we sought a variable independent of dry mass to ascertain rates of incorporation of  $^{15}\text{N}$  to each tissue. Thus, we calculated a ratio of incorporation of  $^{15}\text{N}$  (RI $^{15}\text{N}$ ) by dividing the calculated  $^{15}\text{N}$  of each tissue ( $^{15}\text{N}_{\text{tissue}}$ ) by the expected value of  $^{15}\text{N}$  if the dose were homogeneously distributed ( $^{15}\text{N}_{\text{total}}$ ). If a tissue preferentially incorporates  $^{15}\text{N}$ , then RI $^{15}\text{N}$  is  $>1$ ; if a tissue incorporates less  $^{15}\text{N}$  than expected if the distribution of the dose were homogeneous, then RI $^{15}\text{N}$  is  $<1$ .

#### Statistical analyses

Means are reported  $\pm 1$  s.d. unless otherwise noted. We tested for differences in  $m_b$ ,  $T_b$  and metabolic rates using two-way repeated-measures ANOVA (RM-ANOVA), with 'group' (3% or 20% protein diet) and 'stage' (pre-fast, fast and refeeding) as fixed factors. To test for differences in the isotopic signatures, we used two-way ANOVA with 'group' and 'organ' as fixed factors. When the interaction terms were not significant, we removed them from the analyses and tested for significance of the intercepts. To detect significant differences between groups we performed the Tukey's *post hoc* test. Differences in dry masses of the organs of the birds between dietary groups were tested using Student's *t*-test for independent variables. To detect differences in the relationship between the RI of intestine and muscle between dietary groups, we did analysis of covariance (ANCOVA). However, assumptions of normality and homoscedasticity were not met, even after several different transformations of the data. Therefore, we generated a common line by regression, and calculated the residuals of the regression. Thereafter, we did a *t*-test to compare the residuals of the 3% group and the 20% group. All statistical tests were done with SigmaPlot 11.0 (SYSTAT, San Jose, CA, USA) and SPSS 18.0 (Chicago, IL, USA), with the null hypothesis rejected at  $P \leq 0.05$ .

## RESULTS

We did not find significant differences between sexes for any of the variables we tested ( $P > 0.20$ ); therefore, we pooled the data for subsequent analyses.

#### Body mass, food intake and resting metabolic rates of blackcaps

Body mass of blackcaps changed significantly during the experiment (RM-ANOVA,  $F=216.0$ , d.f.=38,  $P < 0.001$ ). *Post hoc* tests indicated that  $m_b$  decreased after food deprivation (Tukey's test,  $P < 0.049$ ) and remained low thereafter, even after 2 days of re-feeding (Fig. 1A). When we removed a single outlier (a datum larger than 2 s.d. from the mean) from the 20% group, the dry mass of the

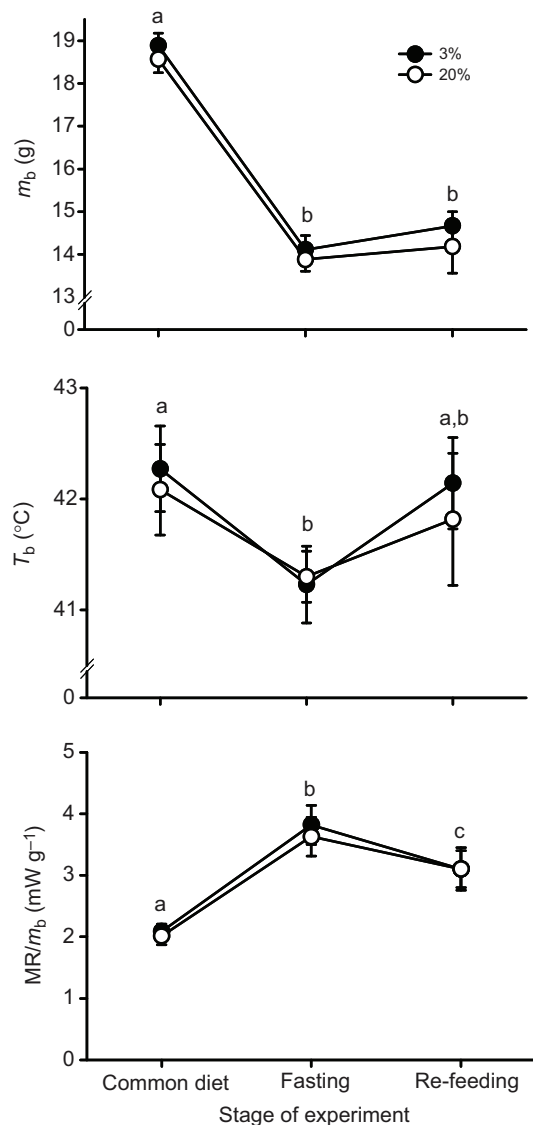


Fig. 1. (A) Body mass ( $m_b$ ), (B) body temperature ( $T_b$ ), and (C) mass-specific metabolic rate ( $\text{MR}/m_b$ ), of captive migrant blackcaps fed a common diet, after 2 days of food and water deprivation, and after 2 days of re-feeding with either a low-protein (3% of dry mass, filled circles) or a high-protein (20% of dry mass, open circles) diet. Body mass and  $T_b$  decreased significantly after fasting, and remained constant thereafter. Metabolic rate increased significantly after fasting, and decreased after re-feeding. There were no significant differences in  $m_b$ ,  $T_b$  or MR between diet groups. Different letters indicate significant differences between stages ( $P \leq 0.05$ ).

intestine was significantly higher in the group fed 20% protein than in the group fed 3% protein ( $t=-2.31$ ,  $d.f.=16$ ,  $P=0.044$ ). Intestine length was not significantly different between diet groups ( $t=-0.49$ ,  $d.f.=17$ ,  $P>0.63$ ). Dry masses of the remaining organs did not differ significantly between diet groups ( $P>0.05$  in all cases). Food intake during the 2 days of refeeding was  $10.6\pm 2.3$  g and  $10.9\pm 4.7$  g in the 3% and the 20% diet groups, respectively, and was not significantly different between diet groups ( $t=0.21$ ,  $d.f.=17$ ,  $P>0.83$ ).

MR and  $T_b$  were significantly lower after food deprivation than before (RM-ANOVA,  $F=5.62$ ,  $P<0.02$  and  $F=4.53$ ,  $P<0.03$ , respectively) (Fig. 1B). MR was not related to  $m_b$  before food deprivation ( $F=0.2$ ,  $P>0.66$ ), and it was isometrically related to  $m_b$  after fasting and after refeeding ( $F=9.9$ ,  $P<0.005$  and  $F=17.7$ ,  $P<0.001$ , respectively). Therefore, we calculated mass-specific MR ( $MR/m_b$ ), which increased after food deprivation and decreased after re-feeding (RM-ANOVA,  $F=24.3$ ,  $P<0.001$ ; Fig. 1C).

### Isotope values

$^{15}\text{N}$  of the different organs was not significantly different between diet groups ( $F=0.58$ ,  $d.f.=1$ ,  $P>0.50$ ). However, organs differed significantly in their isotope values ( $F=27.4$ ,  $d.f.=4$ ,  $P<0.001$ ). We found that pectoral muscle, leg muscle and gizzard had significantly lower isotope values than intestine, which, in turn, had a lower value than liver (*post hoc* Tukey's test,  $P<0.001$ ). We calculated a threshold value ( $^{15}\text{N}_{\text{total}}$ ), specifically, the atom% of  $^{15}\text{N}$ , assuming that the doses of  $^{15}\text{N}$  were homogeneously distributed in the bodies of birds. The  $^{15}\text{N}_{\text{total}}$  threshold was 0.45% and 0.44%, corresponding to  $\delta^{15}\text{N}$  of 226.2 and 209.0 for the groups fed with 3% protein and 20% protein, respectively. Pectoral and leg muscles had isotope values that were significantly below the threshold, whereas the values for intestine and liver were above the threshold for both diet groups ( $t>2.97$ ,  $d.f.=24$ ,  $P<0.01$ ; Fig. 2). Two-way ANOVA indicated significant differences in the ratio of incorporation of  $^{15}\text{N}$  ( $\text{RI}^{15}\text{N}$ ) among tissues ( $F=24.7$ ,  $d.f.=4$ ,  $P<0.001$ ) and between diet

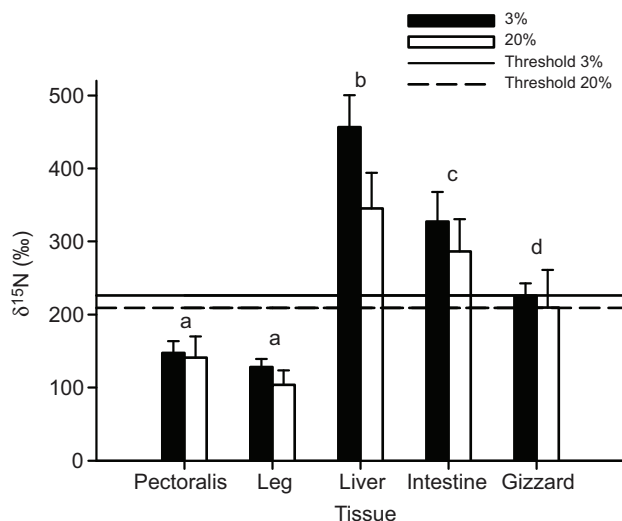


Fig. 2. Comparison of the relative enrichment of  $^{15}\text{N}$  in various tissues of blackcaps fed a low-protein (3% of dry mass, filled bars) or a high-protein (20% of dry mass, open bars) diet. The solid and dashed horizontal lines represent the calculated enrichment based on a null model of homogenous tracer distribution (see Materials and methods for details) for birds fed a low- or high-protein diet, respectively. Exogenous protein was preferentially allocated to the liver and intestine and away from the major muscle groups. There were no significant differences in enrichment between diet groups. Different letters indicate significant differences between tissues ( $P\leq 0.05$ ).

groups ( $F=6.2$ ,  $d.f.=1$ ,  $P<0.02$ ). Specifically, leg muscle, pectoral muscle and gizzard had significantly lower  $\text{RI}^{15}\text{N}$  values than intestine and liver (*post hoc* Tukey's test,  $P<0.001$ ). Birds fed the 3% protein diet had a lower  $\text{RI}^{15}\text{N}$  than birds fed the 20% protein diet.

We found a significant positive correlation between  $\text{RI}^{15}\text{N}$  of pectoral muscle and  $\text{RI}^{15}\text{N}$  of intestine (Fig. 3). Because the assumptions of ANCOVA were not met, we tested for differences in the residuals between the group fed with 3% protein and the group fed with 20% protein and found significant differences ( $t=2.75$ ,  $P<0.03$ ). The regression coefficient was greater in the group fed 3% protein (1.80) than in the group fed 20% protein (1.46). A higher slope in this regression indicates that more N was allocated to rebuild the intestine than was routed to muscle.

### DISCUSSION

A surprising result of our study was the increase in  $MR/m_b$  in blackcaps deprived of food and water for 2 days, especially as their  $T_b$  dropped significantly, as was found in other fasted birds (McKechnie and Lovegrove, 2002; Ben Hamo et al., 2011). In other species of food-deprived birds, RMR decreased more than 50% after migratory flights (Klaassen and Biebach, 1994; Bettley et al., 2000). Indeed, Klaassen and Biebach (Klaassen and Biebach, 1994) found that in fasted garden warblers (*Sylvia borin*), RMR was 64% of that of control birds, but they did not find a relationship between body mass and RMR. In the great knot (*Calidris tenuirostris*), lean  $MR/m_b$  was found to decrease almost 50% after migration, which could be explained by a decrease in the size of the metabolically active organs, or a reduction in tissue-specific MRs (Battley et al., 2001). However, migratory flight in great knots that make a non-stop, 5400 km flight is not comparable to that of blackcaps, which fly a fraction of that distance. Different species of vertebrates increase their MR in response to starvation to increase activity levels to escape unfavorable local conditions (McCue, 2010; and references therein). The blackcaps

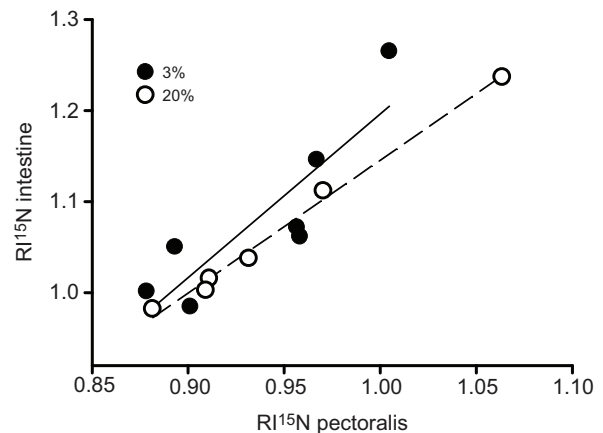


Fig. 3. The relationship between the ratio of incorporation of labeled nitrogen isotope ( $\text{RI}^{15}\text{N}$ ) in the intestine and in the pectoral muscle in captive migrant blackcaps fed a low-protein (3% of dry mass; filled circles, solid line) or a high-protein (20% of dry mass; open circles, dashed line) diet. If a tissue preferentially incorporates  $^{15}\text{N}$ , then  $\text{RI}^{15}\text{N}$  is  $>1$ ; if a tissue incorporates less  $^{15}\text{N}$  than expected, then  $\text{RI}^{15}\text{N}$  is  $<1$ . The slope of the relationship was significantly greater in blackcaps fed a low-protein diet compared with blackcaps fed a high-protein diet, suggesting that birds with less available dietary protein allocated more exogenous (dietary) protein to the intestine than to muscle.

used in our study showed strong migratory restlessness (Zugunruhe) after they were deprived of food, which might account for the observed increase in MR (Aamidor et al., 2011).

In the present study, blackcaps did not increase  $m_b$  appreciably during the first 2 days of re-feeding after simulated in-flight starvation. This is consistent with previous observations in blackcaps subjected to a similar experimental protocol (Karasov and Pinshow, 2000; Aamidor et al., 2011). After the second day of re-feeding,  $m_b$  of blackcaps increased significantly, although  $m_b$  in birds with less protein in their food increased slower than in those eating the high protein diet (Aamidor et al., 2011). Food intake of blackcaps was not different between dietary groups during the first 2 days of re-feeding, suggesting that digestive constraints may limit food intake soon after fasting, as reported by others (reviewed in McWilliams and Karasov, 2005). Changes in intestine mass, but not in length, are consistent with results of Karasov et al. (Karasov et al., 2004). In their study, intestines in fasted blackcaps had shorter villi and showed distal disintegration compared with those of re-fed birds that had intestine morphology similar to control birds fed for the duration of the experiment (Karasov et al., 2004). Therefore, it is possible that differences in the mass of the intestine in the two groups of birds were related to changes in the cellular structure of the intestine (see Starck, 1999).

We found that GIT mass of blackcaps increased faster than did muscle mass during the first days of the refeeding period. Organs of re-fed blackcaps may be divided into three groups according to  $^{15}\text{N}$  enrichment: liver and intestine consistently had the highest tracer enrichment, followed by the gizzard, and tissues not part of the GIT, i.e. pectoral and leg muscles, had the lowest  $^{15}\text{N}$  incorporation. This is consistent with the known patterns of carbon isotope incorporation in the tissues of birds (Bauchinger and McWilliams, 2009). Bauchinger and McWilliams (Bauchinger and McWilliams, 2009; Bauchinger and McWilliams, 2010) analyzed lean tissues, containing negligible carbohydrate levels, for isotopes and suggested that carbon incorporation reflects protein incorporation. We found that the RI, which corrects for differences in organ size, was higher in intestine and liver than in gizzard, pectoral and leg muscles in both dietary groups of blackcaps. Therefore, our data reflect different rates of incorporation of N into tissue. These results support the interpretation of Bauchinger and McWilliams (Bauchinger and McWilliams, 2009; Bauchinger and McWilliams, 2010) that the protein turnover rates of organs influence their extent of phenotypic flexibility in mass.

Our results imply that the birds re-fed with the 3% protein diet incorporated less exogenous N than the birds re-fed with the 20% protein diet. The RI of N was lower in the 3% diet group than in the 20% group. This suggests that either the birds fed with a low-protein diet incorporated less exogenous protein into their tissues, or that the protein in the tissues had a higher residence time (i.e. a lower turnover rate) than in birds re-fed with the high-protein diet. A consequence of a lower RI of N is slower rebuilding of the GIT in the group fed 3% protein. However, we found evidence for compensatory mechanisms that birds may use to rebuild their GIT faster, even when protein is limited. For example, we found a significant positive correlation between the RI of the intestine and that of the pectoral muscle, and differences in the regression coefficient between the groups re-fed on diets with low and high protein content. The slope of this relationship was significantly higher in the 3% group. This finding also rules out the possibility that birds fed the 3% protein diet need a smaller intestine because their food processing requirements are less demanding. If that were the case, the slope of the relationship between the RI of N in intestine

and muscle would be the same for both groups of birds. The difference in slopes suggests that although blackcaps re-fed a low-protein diet incorporated less exogenous N, they allocated a greater amount of the dietary protein to their GIT than to their muscles. However, this mechanism was not sufficient to provide for the rebuilding of the GIT at a rate comparable to those of the birds re-fed with the 20% protein diet.

In conclusion, we found that blackcaps fed less dietary protein during a simulated migratory stopover reconstituted their GIT slower than birds fed more protein. This has straightforward ecological and evolutionary consequences. During migration, birds spend twice as much energy at stopovers than during active flight (Wikelski et al., 2003). Therefore, blackcaps should choose stopover sites and foods at these sites that allow them to maximize protein intake to minimize the time it takes to rebuild their GIT and, thus, resume migration as soon as possible.

#### ACKNOWLEDGEMENTS

The research was done under permit 37066 from the Israel Nature and National Parks Protection Authority and Ben-Gurion University Animal Ethics Committee authorization BGU-R-08-2009 to B.P. We thank Ortal Mizrahy, Miri Ben-Hamo, Darren Burns, Shai Pilosof, Ishai Hoffmann and Marc Goldberg for their assistance in the field and in the laboratory. Two anonymous referees gave insightful comments on an earlier version of the manuscript. This is paper number 761 of the Mitrani Department of Desert Ecology.

#### FUNDING

This project was funded by a US–Israel Binational National Science Foundation grant [no. 2005119 to B.P. and S.R.M.], and Blaustein Postdoctoral Fellowships awarded to A.M.-G. and M.D.M.

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