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Changes in blood stable isotopes ($\delta^{13}C$, $\delta^{15}N$), plasma corticosterone and body mass in exercising birds using a wind tunnel

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Abstract

Blood stable isotope compositions in birds reflect the dietary isotopic signature at the time of the cellular blood synthesis. Several studies suggest that stable isotope ratios of some elements such as nitrogen ($\delta^{15}N$) can change in response to individual metabolic and physiological conditions.

Using a wind tunnel experiment we tested if endurance flight in birds alters the metabolic state and thereby induces changes in blood $\delta^{13}C$ and $\delta^{15}N$ values. We trained European starlings (*Sturnus vulgaris*), that were held under similar diet conditions, to fly in a wind tunnel for up to six hours. While there was a substantial post-flight increase in plasma corticosterone concentrations and a decrease in body weight, we found no significant difference in blood $\delta^{13}C$ and $\delta^{15}N$ values before and after flight. These findings suggest that blood $\delta^{13}C$ and $\delta^{15}N$ values from birds caught in the wild most likely reflect the dietary isotopic sources at the time of blood tissue synthesis even after a previous/recent long-distance migration or endurance exercise.

Introduction

In avian sciences, stable isotope analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) measurements has been widely used to study diet, trophic structure, and nutritional stress. Recently, some laboratory experiments have been conducted to determine how variation in metabolic activity affects stable isotope composition (e.g. Carleton *et al.* 2008; Bauchinger & McWilliams 2009; Bauchinger *et al.* 2010). For instance, using stable-isotope-labeled diets Bauchinger et al. (2010) quantified the rate of carbon turnover in blood cells of zebra finches (*Taeniopygia guttata*) hold in three groups: cold-exposed birds, exercised birds that were flown for 2 hours per day in a flight sector and birds that were not exercised. The results indicate that increases in metabolism associated with cold-exposure or exercise did not produced significant increases in carbon turnover rate in blood. However, the effect of long-distance flight and its associated physiological conditions on stable isotope values in avian tissues have not yet been explored in detail. Therefore, several authors have been calling for more laboratory experiments (e.g. Gannes *et al.* 1997, Phillips *et al.* 2005, Kempster *et al.* 2007).

In a previous experiment, applying an isotopically labeled diet-shift in birds trained to fly in a wind tunnel, we tested the assumption that measurements of $\delta^{13}C$ turnover are likely to represent minimal estimates since wild migratory birds undergo increased metabolism and exercise during migratory flights (Hobson and Yohannes 2005). We found no difference between experimental and control groups in the rate of $\delta^{13}C$ turnover. For both groups, diet was switched from a primarily C-3 content to a C-4 content and blood samples were taken throughout a period of 53 days. Our results supported the contention that blood carbon isotope turnover rates were not different between exercised and non-exercised birds (Hobson and Yohannes 2005). However, in the mentioned previous experiment, we were not able to hold birds at high levels of exercise and we did not analyse

$\delta^{15}N$ turnover rates. On average, birds had been exercised between minimum 35 minutes and a maximum of 67 minutes per day.
In the study at hand, we aimed to extend the flight for up to 6 hours and analyse both $\delta^{13}C$ and $\delta^{15}N$ isotope changes. We used hand-raised European starlings (*Sturnus vulgaris*) (N = 4) and examined whether a controlled non-stop wind tunnel flight lasting up to six hours triggers physiological changes, and whether a shift in cellular blood $\delta^{15}N$ or $\delta^{13}C$ values is observable. Additionally, the extent of physiological activity was measured as concentration changes of the blood plasma hormone corticosterone as well as via pre- and post-flight changes in the bird's body masses.

Potentially, extreme heavy exercise, such as long-distance migration could alter nitrogen use efficiency and result in un-replenished loss of stored nitrogen. If exercise induces increased nitrogen-use efficiency (e.g. as reported by Romano 2000 in dietary restricted animals), we would expect depletion in $\delta^{15}N$ (decreased fractionation), whereas if nitrogen loss exceed nitrogen replacement, enrichment in $\delta^{15}N$ would be expected (increased fractionation and $\delta^{15}N$ values). Food was supplied ad libitum and we did not conduct a diet switch, therefore, a shift in isotope values would not be due to a change in diet. We hypothesized that an observed isotope shift would result from physiological processes due to flight protein metabolism.

Theoretically, higher values of $\delta^{15}N$ and $\delta^{13}C$ are expected because during physiological stress (e.g. nutritional stress) blood is expected to preferentially retain heavy isotopes liberated during protein catabolism (Hobson *et al.* 1993; Cherel *et al.* 2005). Yet, in these catabolism studies, it is shown that substantial shift in isotopes were observed only after several days to weeks of starvation or nutritional stress. This suggests that the nitrogen balance requires sufficient loss of nitrogen from the body pool to achieve a significant shift in isotopes in blood (Martinez &Wolf 2005).

However, in the current experiment, we aimed to simulate physiological activity similar to long-distance migratory flight using a wind tunnel exercise of up to 6 hours. Therefore, the birds were not hold in nutritional stress and we measured the tissues of birds sampled immediately before and after the exercise. Simply, we were interested to evaluate whether blood assays of wild migrating birds exposed to long distance flight exhibit honest isotopic signals of diet.

Methods

Wind tunnel experiment

Birds were taken as nestlings from natural breeding colonies in Upper Bavaria in spring 2005 and hand-raised. After the completion of post juvenile moult the birds were housed in groups of 4 individuals in aviaries (approx. 150×200×200 cm) adjacent to the flight chamber of a low-turbulence wind tunnel specifically designed for bird flight experiments at the Max-Planck Institute for Ornithology, in Seewiesen, Germany. First, birds were conditioned to the flight chamber and the sound of the operating wind tunnel and they learned to enter and exit the flight chamber. After that, birds were trained to fly singly or in groups of up to five birds at various wind speeds for increasing time periods. Training was completed when the birds were able to fly for up to six hours at a wind speed of 12 m/s.

From November on, all birds received the same standard diet (a mixture of dried insects, heart, rusk, curd, and chicken egg, supplemented with vitamins, minerals (AviConcept®), fresh fruit and salad and Realpasto®). Samples of the diet were collected and frozen for stable isotope analysis (see below).

Blood corticosterone level and stable isotope analyses

Experimental flights started in December 2005. A first blood sample and body mass measurements (to the nearest 0.1g) were taken from individual birds either one day before the experimental flights started or immediately after lights-on on the day of the experiments. The samples were used to establish pre-flight (or resting) levels of corticosterone, blood carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) signature and body mass. Birds were let to fly in the wind tunnel at a wind speed of 12 m/s. Samples were collected from four different individuals with three different flight durations ranging from 100 to 360 minutes (two starlings flew 360 minute, while two others flew 285 and 100 minutes non-stop. Within less than 3 minutes after the experiment a post-flight body mass measurement and blood samples were taken for blood $\delta^{13}C$ and $\delta^{15}N$ isotope and plasma corticosterone analysis. Blood samples (approx. 100 µl) were taken from the wing vein using 23-gauge needles and collected in heparinised capillaries. Plasma was immediately separated by centrifugation with a Compur Minicentrifuge and kept at -70°C until analysis. Corticosterone concentrations were assessed by radioimmunoassay (RIA) (Goymann *et al.* 2006). Each sample was run in duplicates. The detection limit of the assay was 5.81 pg/ml, the intra-assay coefficient of variation was 8.0%.

Sub-samples of ca. 0.35 mg of dry cellular blood from each bird and diet samples were weighed in small tin cups to the nearest 0.001 mg, using a micro-analytical balance. Samples were then combusted in a Eurovector (Milan, Italy) elemental analyzer at Kompetenzzentrum Stabile Isotope, Universität Göttingen, Germany. The resulting N_2 and CO_2 gases were separated by gas chromatography and admitted into the inlet of a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. Measurements are reported in δ-notation relative to the PDB for Carbon and atmospheric N_2 standard in parts per thousand deviations (‰). Typical precision of analyses was ± 0.2 ‰ for $\delta^{15}N$ and ± 0.1 ‰ for $\delta^{13}C$. The standard for $\delta^{15}N$ is atmospheric nitrogen and Vienna Peedee belemnite (VPDB) for $\delta^{13}C$. Sample analysis included one in-house standards (acetanilide) for every10 samples, which has been calibrated against NIST or other certified standards. Based on several hundreds of replicate assays of internal laboratory standards indicate measurement errors (SD) of ± 0.1 -0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Statistics

Descriptive statistics were used to summarize differences in measured body weights, stress hormone levels and stable isotope values.

The proportional change in the different variables was calculated as:
proportional change = [(post-flight value − pre-flight value)/post-flight] × 100.

Differences between pre- and post-flight scores of the quantitative variables were compared using the parametric paired t-test, after confirming normal distribution. Power analysis was used to estimate the required minimum sample size to values detect as significantly different in the paired sample test. Data analysis, statistics and graphical presentation were performed using the software package R for Windows 2.9.1 (R Core Development Team 2009) and standard procedures.

Results

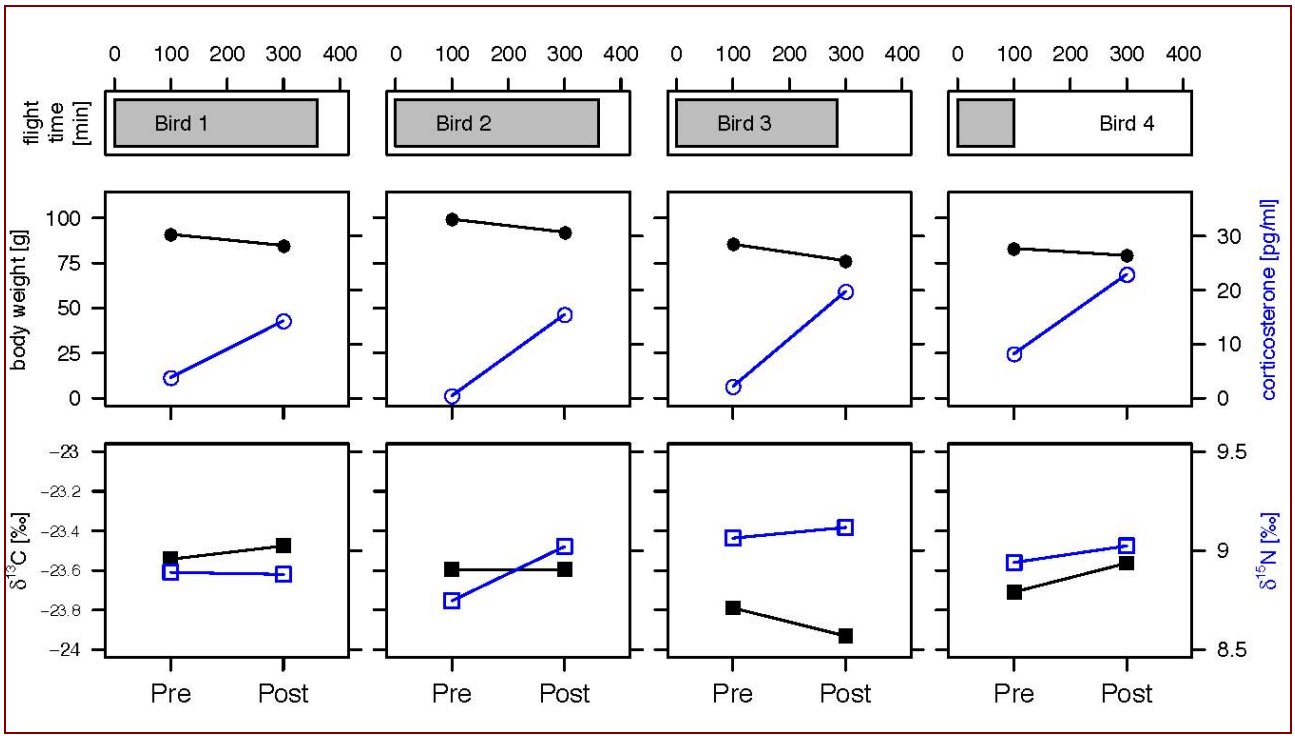
The exercise in the wind tunnel treatment induced an effect on the physiological conditions in terms of body mass and hormone corticosterone level of the starlings (Figure 1). All birds weighed significantly less (Student's t-test: t_3= 6.15, p = 0.008) and lost up to 8 g of the initial (pre-flight) body mass after the endurance flight (Table 1). Post-flight increase in corticosterone level reached up to 18 pg/ml higher concentrations compared to the pre-flight level and was significantly elevated (Student's t-test: t_3 = -9.83, p = 0.002; Figure 1). There was no linear pattern in relative stress level increase in relation to increase in flight time, neither for body mass change.
We found no evidence of shift in $\delta^{15}N$ (Student's t-test: t_3 = -1.64, p = 0.20) or $\delta^{13}C$ (Student's t-test: t_3 = -0.29, p = 0.79) in response to the simulated long-distance flights in the wind tunnel (Figure 1).

Despite that all variables taken into account might be biased due to a small sample size (N =4) and the lack of a control group. Nevertheless, the qualitative direction of responses to the experiment coincide between the four birds and each bird acted as its own control by taking measurements pre- and post-treatment.

Flight	Body mass (g)	Hormone (pg/ml)
pre	88.54	3.48
post	81.92	18.13
% change	-8.13	80.95

Table 1 Summary of mean body weight and hormone before/after flight and proportion of change in body mass in relation to flight time

Figure 1. Flight duration, body weight, corticosterone, $\delta^{13}C$ and $\delta^{15}N$ values of four birds measured before and after flight in wind tunnel. *Upper panel* flight times in minutes middle panel: change in body weight (black lines) and corticosterone levels (blue lines). *Lower panel* change in $\delta^{13}C$ (blue lines) and $\delta^{15}N$ (black lines) values before and after the experiment



Discussion

Although these results should be interpreted with caution due to the low sample size, the most parsimonious result we obtained from this wind tunnel experiment is that exposure to non-stop flight caused a decrease in body mass and a rapid increase in stress hormone level, but no substantial shifts in $\delta^{13}C$ or $\delta^{15}N$ isotopic compositions were detected. This suggests that after a flight duration of up to six hours physiological effects are not able to induce isotopic changes in the blood (of migrating or exercising bird).

As glucocorticoids such as corticosterone are involved in the physiological cascade responsible for mobilizing glucose stores in preparation for physical activity (Coleman *et al.* 1997), the increase in corticosterone may reflect increased metabolic activity as a result of the exercise. However, it could well be that a threshold exercise level and body mass loss or level of metabolic activity might exist below which a shift in $\delta^{13}C$ and $\delta^{15}N$ is negligible. This is expected when the activity levels the birds experienced by the wind tunnel exercise did not trigger substantial internal cycling of nitrogen (and isotopic enrichment) with a significant loss of depleted isotopes.
So far, experimental investigations dealing with physiological condition and isotopic signatures have mainly concentrated on exposure to cold, food restricted, starving or fasting organisms. It has been reported that tissues of starving or fasting animals show a progressive increase in $\delta^{13}C$ and $\delta^{15}N$ in relation to body mass decrease (Hosbson *et al.* 1993, Cherel *et al.* 2005). The wind tunnel treatment induced a rapid decrease in body mass, which could be due to a rapid loss of water and fat (Engel *et al.* 2006). However the blood isotope values remained stable. This indicates that short-term body mass loss such as those associated with long distance flights (e.g. during migration: for up to 8% in this study experimental species) does not cause a substantial immediate shift in blood isotope signatures.

Hatch *et al.* (1995) reported an immediate detection of catabolic state via change in $\delta^{13}C$ of blood proteins in growing chicks. In our study, the variability present in isotope values of exercising individuals was surprisingly small. All blood $\delta^{13}C$ or $\delta^{15}N$ ranged within a –23.8 ‰ to –23.5‰ and –23.9 ‰ to –23.5‰ ($\delta^{13}C$) and 8.7‰ to 9.0‰ and 8.9‰ and 9.1‰ ($\delta^{15}N$) pre-and post-flight; respectively, which suggests that the duration of activity the birds were exposed to in this study does not induce a significant variation in $\delta^{13}C$ or $\delta^{15}N$. This could be attributed to the fact that in our study we analyzed isotopic signatures of blood cells that are known to have longer turnover rates with up to 17-21 days (Bauchinger &McWilliams 2009). Future analyses of similar experiments that apply tissues with faster turnover rate such as breath or plasma are necessary to draw conclusions on the effect of physiological stress on such tissue isotopes (e.g. Hatch *et al.* 2002).

We also note that the starlings in our study were exposed to less extreme but biologically meaningful level of exercise in wind tunnel. Both, our previous wind tunnel experiment and the current study showed that both blood carbon and nitrogen isotope does not differ between exercising and non-exercising birds. These results demonstrate that blood isotope values of birds reflect mainly the isotopic composition of the dietary sources at the time of blood tissue synthesis. This has the important relevance for ecological research that, for instance, during long flight duration and migration where birds experience a rapid loss in body mass, (e.g. Yohannes *et al.* 2008), the isotopic values do not necessarily reflect the nutritional and physiological condition. Consequently, the analysis of blood isotopes in birds that are exposed to exercise is not a very sensitive method to determine immediate catabolic changes in carbon and nitrogen isotopes.

In summary, our experiment confirms that avian blood $\delta^{13}C$, $\delta^{15}N$ reflect the isotopic composition of the dietary sources at the time of blood synthesis; even when the animal's metabolic system might be challenged by long distance flight. This experiment is the first to document the relation of corticosterone, body mass and blood tissue isotope in controlled experiments in four birds that are conditioned to fly in a wind tunnel for a maximum of 6 hours. Our experiment was conducted in a literally closed system and did not provide the condition for a diet-switch and a significant isotopic mass balance to take place; which would require depletion in one

component and enrichment in another for any isotopic change to take place. However, in natural systems birds perform long, non-stop flight without being exposed to isotopically different diet en route. Future analyses of similar experiments that consider tissues that have a faster turnover rate (e.g. breath, plasma) coupled with individual nitrogen balance in relation to exposure to varying exercise levels are necessary to draw firm conclusions regarding the effect of physiological conditions on tissue isotopes. Such experiments should also consider applying longer flight duration, diet switch and a larger sample size of birds.

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