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RESEARCH ARTICLE

Physiological effects of increased foraging effort in a small passerine

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ABSTRACT

Foraging to obtain food, either for self-maintenance or at presumably elevated rates to provide for offspring, is thought to be an energetically demanding activity but one that is essential for fitness (higher reproductive success and survival). Nevertheless, the physiological mechanisms that allow some individuals to support higher foraging performance, and the mechanisms underlying costs of high workload, remain poorly understood. We experimentally manipulated foraging behaviour in zebra finches (*Taeniopygia guttata*) using the technique described by Koetsier and Verhulst (2011). Birds in the 'high foraging effort' (HF) group had to obtain food either while flying/hovering or by making repeated hops or jumps from the ground up to the feeder, behaviour typical of the extremely energetically expensive foraging mode observed in many free-living small passerines. HF birds made significantly more trips to the feeder per 10 min, whereas control birds spent more time (perched) at the feeder. Despite this marked change in foraging behaviour, we documented few short- or long-term effects of 'training' (3 days and 90 days of 'training', respectively) and some of these effects were sex specific. There were no effects of treatment on basal metabolic rate, haematocrit, haemoglobin or plasma glycerol, triglyceride and glucose levels, and masses of kidney, crop, large intestine, small intestine, gizzard and liver. HF females had higher masses of flight muscle, leg muscle, heart and lung compared with controls. In contrast, HF males had lower heart mass than controls and there were no differences for other organs. When both sexes were pooled, there were no effects of treatment on body composition. Finally, birds in the HF treatment group had higher levels of reactive oxygen metabolites (dROMs) and, consequently, although treatment did not affect total anti-oxidant capacity, birds in the HF treatment group had higher oxidative stress.

KEY WORDS: Exercise physiology, Workload, Oxidative stress, Energetics, Body composition, *Taeniopygia guttata*

INTRODUCTION

Foraging to obtain food is essential for successful reproduction and survival. However, foraging in many animals, either for self-maintenance or at presumably elevated rates to provide for offspring, is thought to be an energetically demanding activity that should select for high workload ability (Bryant and Tatner, 1991; Maurer, 1996; Piersma and van Gils, 2010). Strong selection would be expected to decrease variation in traits underpinning foraging but we see considerable individual variation in foraging and provisioning effort (Royle et al., 2014; Fowler and Williams, 2015). This suggests that although some individuals might have higher foraging ability the high workload associated with foraging and provisioning is costly, which would oppose directional selection. In support of this view, Mariette et al. (2011) found that wild breeding zebra finches (*Taeniopygia guttata* Reichenbach 1862) covered an average of 6.4 km daily to forage for food, but some individuals travelled up to 19.4 km and these 'hard-working' individuals appeared to pay a cost in that they took longer to re-nest after a successful breeding attempt. Although there is some experimental evidence from studies directly manipulating foraging costs, or demand via brood size manipulation, that increased workload leads to reduced fecundity (Veasey et al., 2001; Simons et al., 2014) and increased mortality (Daan et al., 1996), the physiological mechanisms that allow some individuals to support higher foraging performance, and the mechanisms underlying costs of high workload, remain poorly understood.

Exercise can be broadly defined as any behaviour that elevates the level of intensity of activity or workload, in response to an ecological demand for increased performance (Booth et al., 2012; Halsey, 2016; Irschick and Higham, 2016). Hence, given the high activity level and metabolic demand associated with foraging flights (Maurer, 1996), and the intuitive, positive relationship between foraging performance and fitness during chick-rearing, it might be valuable to apply an exercise perspective on workload during foraging and parental care (Williams and Fowler, 2015). The physiology of exercise has been investigated in many model systems, e.g. migratory birds flying in wind tunnels (Guglielmo, 2010; Price et al., 2010) and exercise training in captive birds using automated systems (Nudds and Bryant, 2000; Costantini et al., 2012; Zhang et al., 2015). Although these model systems might provide a good starting point for understanding physiological adaptations of aerobic capacity associated with exercise or workload, the critical relationship in free-living animals between exercise and acquisition of resources is often ignored in these studies, many of which also use using forced exercise protocols. Specifically, in relation to foraging it is of great importance to adopt an exercise contingent method, where animals have to work for food, because the physiological effects of voluntary exercise with access to resources might be very different from those induced by forced exercise in less ecologically relevant contexts (Fonseca et al., 2014; Irschick and Higham, 2016). For instance, Fonseca et al. (2014) found that when acquisition of food was contingent upon the distance rats need to run, adipose tissue was significantly decreased, compared with rats in which food acquisition was not dependent upon running distance. It is also important to consider the relative energetic cost of different types of flight and foraging mode. For instance, some birds use more energetically expensive flapping/

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hovering flights during foraging, while others use less energetically costly soaring flights during foraging (Norberg, 1996). Small passerines search for, and capture, insects during short flights or quick hovers, which has been suggested to be an extremely energetically expensive foraging mode [with a scaling exponent of daily energy expenditure (DEE)=mass$^{1.99}$, as opposed to scaling exponents of DEE=mass$^{0.66–0.75}$ in birds that do not engage in this kind of foraging mode; Tinbergen and Dietz, 1994]. Furthermore, the duration of exercise training can also influence the physiological response of exercise. Most studies only looked at acute physiological effects of exercise, and long-term physiological adjustments have rarely been considered. Koetsier and Verhulst (2011) and Simons et al. (2014) addressed this issue of the influence of food availability on exercise and workload by using a technique to manipulate foraging effort in birds. Their technique forces birds to hop to and hover briefly in front of the feeder to obtain seeds, mimicking the energetically expensive foraging mode of small passerines described above (Tinbergen and Dietz, 1994).

Koetsier and Verhulst (2011; see also Simons et al., 2014; Briga et al., 2017) showed that experimental manipulation of foraging costs affected energy expenditure, survival (individuals reared in experimentally enlarged brood only), and reproduction, but the physiological basis of these effects remains unknown. The objective of our study was therefore to investigate physiological effects of training for increased foraging effort. As animals appear to be able to regulate individual components of their physiology independently (Buehler et al., 2012; Williams and Fowler, 2015), we measured multiple physiological traits: basal metabolic rate (BMR), haematocrit (Hct), haemoglobin (Hb), body composition, glucose, glycerol, triglyceride and oxidative stress. We predicted that in response to high foraging effort treatment, birds would: (1) adopt an energetically costly foraging mode, have higher flight activity and decrease BMR (Koetsier and Verhulst, 2011), (2) elevate Hct and Hb (Fair et al., 2007) in the short term but decrease Hct and Hb eventually when foraging costs become too high and maintaining energy balance becomes more difficult, (3) have enlarged metabolic machinery organs and food processing organs (Swallow et al., 2010) despite an overall decrease in energy expenditure (Westerterp et al., 1994; Wiersma and Verhulst, 2005; but see Williams and Vézina, 2001; Zhang et al., 2015), (4) show increases in markers of energy supply such as triglyceride (Kern et al., 2005), but also (5) show increased levels of oxidative stress (Costantini et al., 2012; Jenni-Eiermann et al., 2014).

MATERIALS AND METHODS

Animal husbandry

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14 h:10 h light:dark, lights on at 07:00 h). All birds were provided with a mixed seed diet [*Panicum* and white millet (1:3), 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass], water, grit (coral sand) and cuttlefish bone (calcium) *ad libitum*, and received a multi-vitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit (no. 1074B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

Experimental manipulation of foraging costs

Foraging costs were experimentally manipulated in a 'high foraging effort' (HF) group using the technique described by Koetsier and Verhulst (2011). Food (mixed seed) was provided in transparent Plexiglas containers (length×width×height: 40×10×13 cm) suspended from the roof of the cage (length×width×height: 122×46×41 cm), with feeding holes low on the front panel to allow access to seeds. Perches made of wooden pencils (diameter 0.8 cm) were fitted adjacent to feeding holes to allow birds to perch while foraging for 21 days prior to the start of the experiment (similar to standard feeders in control cages). We also measured BMR and collected blood samples during the 21-day period, prior to shortening the perches. Over a 14-day period, perches were gradually shortened (0.5 cm every 2 days) and eventually removed completely to train birds to modify their foraging behaviour and obtain seeds in the high foraging cost condition. As the perches became shorter the birds were unable to perch and had to obtain seeds either while flying/hovering in front of the suspended feeder, or by making repeated hops or jumps from the ground up to the feeder (the vertical distance between the cage floor and the feeding holes was ∼30 cm). To prevent birds from eating seeds spilled on the cage floor, the metal tray was removed from the bottom of all HF cages, so that seeds fell through the cage bottom. In lieu of the metal tray, small resting platforms made of egg carton were secured to each side of the cage to allow birds to rest when not foraging. Birds in control foraging conditions (CTR) were given standard feeders (seed fountains) with perches adjacent to them throughout the experiment. A total of four HF cages and four CTR cages were used for the experiment and both HF and CTR conditions were offered simultaneously during the experiment. A picture of the set-up of the HF cage is provided (Fig. S1). Several notable differences between the set-up of this experiment and the set-up in Koetsier and Verhulst (2011) include: (1) the size of the cage is smaller than the aviaries used by Koetsier and Verhulst (2011), and as a consequence the distance birds had to fly for food is presumably smaller, and (2) the aviaries used by Koetsier and Verhulst (2011) were outdoor, i.e. at lower and fluctuating ambient temperatures, while birds in this experiment were housed in temperature-controlled indoor facilities.

Experimental timeline

Male and female birds were housed in groups of eight, in single-sex cages during the experiment and were kept in their respective foraging condition (HF and CTR) for 90 days. To ensure sufficient sample size, the main experiment was repeated over two trials: trial 1 (summer 2014) and trial 2 (spring 2015) with all birds exposed to the same experimental conditions and protocols, as well as environmental conditions in both trials. Birds were randomly assigned to HF and CTR conditions. Specifically, birds from the same home cages were distributed across both treatments, and each treatment consisted of more than one cage. For example, the first bird caught was placed in a HF cage, the second bird caught was placed in a CTR cage, the third bird caught in another HF cage, and so on. Hence, both high- and low-quality birds should be at least somewhat evenly distributed across both treatment groups. We measured BMR and collected blood samples at three time points: (1) prior to the start of the 14-day perch shortening period (pre-treatment), (2) ∼3 days after complete removal of perches (day 3) and (3) ∼60 days after complete removal of perches (day 60) to assess both short- and long-term responses to change in activity level. Birds were kept in their respective foraging conditions for an additional 30 days after the last BMR measurement, at the end of which they were killed and tissues and blood samples were collected for further analysis (day 90). A summary of the experimental timeline is provided in Fig. 1.

Behavioural observations

After completion of all BMR measurements at day 60 (see below), we video-recorded behaviour of birds in each treatment cage for a

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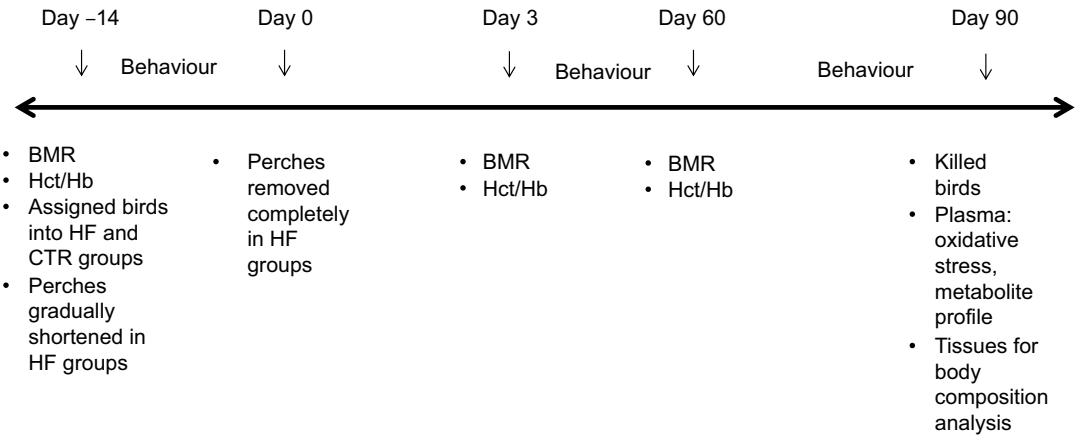

Fig. 1. Summary of the experimental timeline. HF, high foraging group; CTR, control birds; BMR, basal metabolic rate; Hct, haematocrit; Hb, haemoglobin.

total duration of 30 min between 09:00 h and 15:00 h. Individual birds could be identified using a unique combination of colour leg bands. Behaviours quantified during the entire 30 min duration include total time spent foraging, resting and engaging in other physical activities (e.g. preening, perch hop, displacement behaviour, etc.). In addition, similar to Koetsier and Verhulst (2011), foraging flight activity (trips to feeder) was scored for individual birds for a period of 10 min. All behaviour was scored by a single researcher (K.C.H.).

Basal metabolic rate measurement

All BMR measurements were conducted using a flow-through respirometry system (Sable Systems International, Las Vegas, NV, USA) similar to that described in Salvante et al. (2010). O_2 and CO_2 analyzers (Sable Systems International FC-1 and CA-1, respectively) were calibrated every day using standard air containing 20.8% O_2 and 1.10% CO_2. To ensure post-absorptive state at the time of BMR measurement, individuals undergoing metabolic measurement that night were fasted for 3 h before entering the metabolic chambers (Secor, 2009; Salvante et al., 2010). Birds were taken from their cages at 21:00 h and placed in one of four metabolic chambers (1.5-litre stainless-steel coffee canisters, Great Canadian Superstore, Coquitlam, BC, Canada) for 2 h prior to the beginning of measurements. The system was checked for leaks before each round of metabolic rate measurement. All metabolic chambers were placed in an incubator (PTC-1 Peltier effect temperature-controlled portable cabinet, Sable Systems International) maintained at 36°C for the entire duration of BMR measurement, within the thermoneutral zone of the zebra finch (Marchall and Prinzinger, 1991). Each metabolic chamber continuously received ∼500 ml min^{-1} of dry air (using magnesium perchlorate as scrubber). Each of the three metabolic chambers containing a bird and an empty chamber sampling baseline ambient air were sampled for 10 min by a multiplexer (TR-TM4, Sable Systems International) every 40 min, allowing a total of 100 min of recording per chamber over 7 h. BMR calculations were done based on the lowest averaged 5 min of oxygen consumption per measurement sequence according to Lighton's eqns 10.6 and 10.7 (Lighton, 2008) with ExpeData software, version 1.2.6 (Sable Systems International). Birds were weighed immediately before and after measurement and the average of the two masses was used in BMR analysis. Birds were taken out of the metabolic chambers at 06.00 h the next morning.

Physiological measurements and assays

Pre-treatment, day 30 and day 60 blood samples (∼100 µl) were obtained from the brachial vein following puncture with a 26G needle, and blood was collected using a 75 µl microhaematocrit tube. Hct (percent packed cell volume) was measured with digital callipers (±0.01 mm) following centrifugation of whole blood for 3 min at 13,700 ***g*** (Autocrit Ultra 3; BD Diagnostic Systems, Sparks, MD, USA). Hb (g dl^{-1} whole blood) was measured using the cyanomethaemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; BioTek Instruments, Winooski, VT, USA), using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941; Sigma-Aldrich Canada, Oakville, Ontario, Canada) with absorbance measured at 540 nm. Intra- and inter-assay coefficients were 3.1 and 3.8%, respectively. Blood glucose was also measured in individuals at the time of blood sampling using a glucose meter (Accu-Chek Aviva; Roche Diagnostics, Mannheim, Germany).

Blood samples collected at day 90 were assayed for total antioxidant capacity (µmol HClO ml^{-1}; OXY), reactive oxygen metabolites (mg H_2O_2 dl^{-1}; dROMs), and plasma glycerol and triglyceride, in addition to Hct, Hb and glucose. Not all samples were assayed for all measures owing to insufficient plasma volumes, and haemolyzed and lipolyzed plasma samples were excluded (final sample sizes are listed in Table S1). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave 340) and 96-well microplates. Free glycerol and total glycerol were assayed via sequential colour end-point assay (Sigma-Aldrich Canada), using 5 µl of plasma with 240 and 60 µl of glycerol reagent and triglyceride reagent, respectively, with a reading taken at 540 nm after 10 min of incubation at 37°C after the addition of each reagent. Plasma triglyceride concentration was calculated by subtracting free glycerol from total glycerol. The intra-assay coefficient of variation was 4.8%. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (2011), with slight modification. Specifically, we measured dROMs and OXY using the commercial kits dROMs and OXY-Adsorbent Test (Diacron International, Grosseto, Italy), respectively. Intra-assay coefficients for OXY and dROMs were 3.8 and 2.4%, respectively.

Determination of immediate food consumption, dissection and body composition analysis

At 90 days birds were killed by exsanguination under anaesthesia (0.05 ml of 20 mg ml^{-1} xylazine and 0.05 ml of 100 mg ml^{-1} ketamine) and tissues were collected for further analysis. To

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determine immediate food consumption, we collected and weighed seeds from each bird's oesophagus at the time of tissue collection. After dissection, a sample of the right pectoralis muscle was immediately removed and weighed to be used as part of another study. The rest of the carcass was stored at −20°C until all the birds had been killed for further processing. The following tissues were dissected out from each bird: flight muscle (including the supracoracoideus and left pectoral muscle), leg muscle, crop, large intestine, small intestine, gizzard, heart, lungs, liver, kidney and reproductive organs (testes from males; ovary, ovarian follicles and oviduct from females). The presence of yolky follicles allowed us to determine the reproductive state of birds, and birds that were found to be in breeding condition (six females in trial 1, and seven females in trial 2) were excluded from subsequent analysis. Tissues were dried at 60°C for 24 h, weighed (mg, ±0.0001), and the final mass is reported as dry mass.

Statistical analyses

Analyses were carried out using R version 0.99.467 (R Core Team, 2013). Data were first examined for normality using the Shapiro–Wilk test, and data were either transformed prior to analysis or analyzed using a non-parametric test (independent two-group Mann–Whitney *U*-test). For repeated measures analysis (body mass, BMR, Hct, Hb and glucose), we used the lme4 package (Bates et al., 2013) with sex, time and treatment as main effects, and individual bird identity (ID) as a random factor. Trial was initially included in all models but was taken out because we did not detect any main effects of trial, nor interactions between trial and other variables (P>0.1 in all cases). F-statistics and P-values were generated using the lmerTest package (Kuznetsova et al., 2013). Tukey's honest significant difference test (package multcomp; Hothorn et al., 2008) was used to evaluate pairwise comparisons between treatments and time points following a significant mixed model. Additionally, we also ran the repeated measures analysis (body mass, BMR, Hct, Hb and glucose) with day 3 and day 60 time points and treatment as main effects, pre-treatment values as covariate, and induvial bird ID as a random factor. For body composition, OXY, dROMs, triglyceride and glycerol analyses, we used a general linear model (GLM) testing for the effects of sex, treatment, and sex×treatment. To control for the effect of body mass on tissue mass, we used non-reproductive dry body mass (total dry body mass minus dry masses of reproductive organs) as a covariate. In addition, to account for part-whole correlation (Christians, 1999), we subtracted the mass of the tissue used as the dependent variable from the covariate. For instance, the model for testing the effect of treatment on heart mass would read 'heart mass~treatment+(body mass – heart mass)'. Furthermore, to investigate whether there was a treatment effect on dROMs after controlling for total anti-oxidant capacity, we conducted additional analysis by including OXY as a covariate in the model. We report the Z-statistics and the associated P-values. A summary of all data and statistical analyses is provided in Table S2.

RESULTS

Effects of foraging treatment on behaviour and food consumption

When comparing foraging flight activity, HF birds made significantly more trips to the feeder per 10 min (W_{54}=215, P<0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d=1.05; Fig. 2A). Conversely, CTR birds spent more time (perched) at the feeder than HF birds (W_{54}=452.5, P<0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d=0.74; Fig. 2B). There was no significant treatment effect for time spent resting (Z_{54}=1.10, P=0.27) or time spent engaging in other activities (Z_{54}=−1.48, P=0.14). HF birds had ~50% more seeds in their oesophagus at the time of tissue collection than CTR birds (W_{54}=214, P<0.01, Mann–Whitney–Wilcoxon rank sum test, Cohen's d=0.30; Fig. 2C). It should be noted that the order in which birds were was randomised and hence approximately the same number of birds in each treatment group was killed in the morning and in the afternoon.

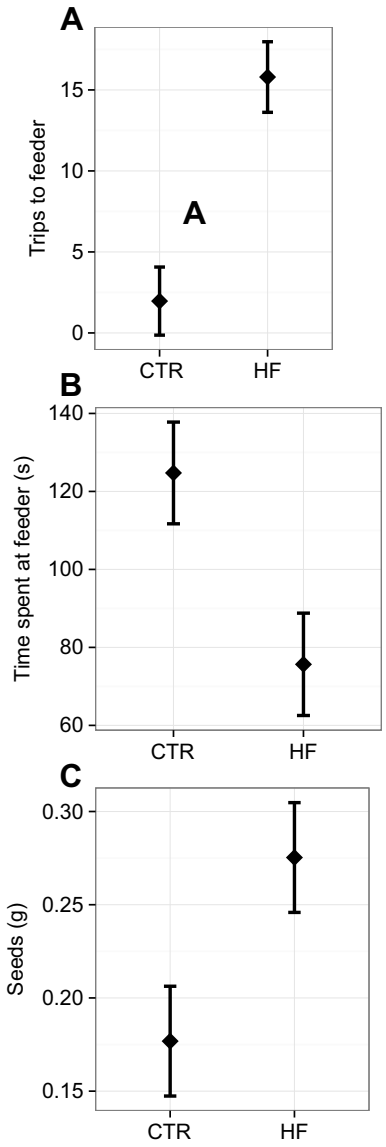

Fig. 2. Effects of foraging treatment on behavior and food consumption. The high foraging cost treatment significantly increased (A) the number of trips birds made to the feeder and (C) immediate food consumption (i.e. dry mass of seeds in the birds' oesophagus at the time of tissue collection), but decreased (B) time spent at feeder (per 1800 s). HF, high foraging group; CTR, control birds. Data shown are least-squared means±s.e.m.

Effects of foraging treatment on body mass, BMR and haematology

Sex was not included in the overall model as there was no significant sex effect or sex×treatment interaction for body mass, BMR and haematology. There was a significant treatment×time interaction for body mass ($F_{2,108}$=4.50, P=0.01) (Fig. 3A). Body masses of HF birds were significantly lower than CTR birds at day 3 (t_{41}=2.23, P=0.03), but in HF birds there was only a marginally significant

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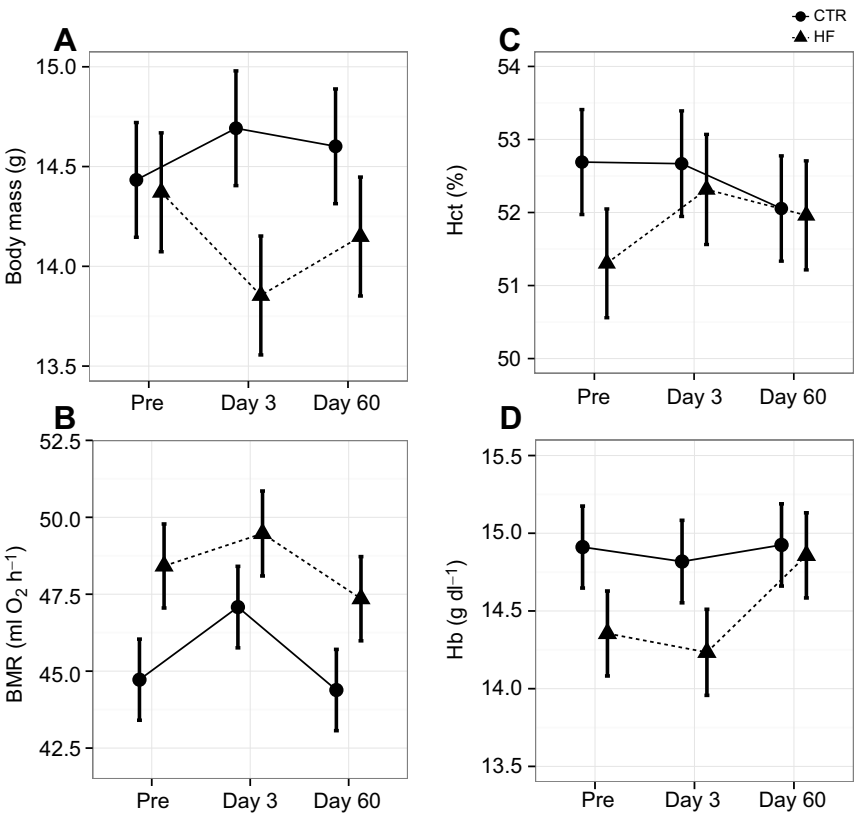

Fig. 3. Effects of foraging treatment on body mass, basal metabolic rate and haematology. The high foraging cost treatment did not affect (A) body mass, (B) basal metabolic rate (BMR), (C) haematocrit (Hct) and (D) haemoglobin (Hb). Filled circles and continuous lines represent control birds; filled triangles and dashed lines represent HF birds. Data shown are least-squared means±s.e.m.

decrease in body mass between pre-treatment and day 3 time points (P=0.07; Fig. 3A). There was no treatment×time interaction for BMR ($F_{2,107}$=0.14, P=0.87; Fig. 3B), Hct ($F_{2,107}$=1.16, P=0.31; Fig. 3C) and Hb ($F_{2,107}$=1.09, P=0.34; Fig. 3D) and no main effect of treatment. It should also be noted that there appear to be small to moderate differences between pre-experimental values between treatments (Cohen's d ranges from 0.04 to 0.49), although none of the differences was significant (P>0.05 in all cases). None of the differences between pre-experimental values between treatments observed could be attributed to sex differences (sex×treatment interactions, P>0.05 in all cases). Similar results were found even when the models were run using day 3 and day 60 time points and treatment as main effects, pre-treatment values as covariate, and individual bird ID as a random factor (Fig. S3).

Effects of foraging treatment on body composition

When both sexes were pooled, there was no significant treatment effect at day 90 for dry mass of organs related to aerobic and metabolic capacity: flight muscle (Z_{52}=−1.59, P=0.11, Cohen's d=0.35), leg muscle (Z_{52}=−7.93, P=0.43, Cohen's d=0.29), heart (Z_{52}=0.41, P=0.68, Cohen's d=0.12) and lungs (Z_{52}=−1.59, P=0.11, Cohen's d=0.44). However, there was a significant sex×treatment interaction at day 90 for dry mass of organs related to aerobic and metabolic capacity: flight muscle (t=−1.80, P=0.05), leg muscle (t=−2.40, P=0.02), heart (t=−2.58, P=0.01) and lungs (t=−2.61, P=0.01). HF females had higher flight muscle mass (Z_{52}=−3.26, P<0.01, Cohen's d=1.06; Fig. 4A), leg muscle mass (Z_{52}=−2.38, P=0.02, Cohen's d=1.32; Fig. 4B), lung mass (Z_{52}=−3.15, P<0.01, Cohen's d=1.30; Fig. 4C) and heart mass (Z_{52}=−0.20, P=0.05, Cohen's d=0.74; Fig. 4D) compared with controls (Fig. S2). In contrast, HF males had lower heart mass (Z_{52}=2.02, P=0.04, Cohen's d=0.50; Fig. 4D) than controls and there were no differences for other organs (P>0.05 in all cases; Fig. S2). Dry mass of kidneys (T=0.73, P=0.47) and food-processing organs – crop (T=−0.54, P=0.60), large intestine (T=−0.59, P=0.56), small intestine (T=−1.09, P=0.28), gizzard (T=0.10, P=0.92) and liver (T=−1.02, P=0.31) – were not affected by HF treatment in either sex (Fig. S2).

Effects of foraging treatment/effort on plasma metabolites and oxidative stress

HF treatment did not influence levels of blood glucose ($F_{2,53}$=2.22, P=0.11; Fig. 5C), plasma glycerol (Z_{35}=−0.57, P=0.57; Fig. 5A) and triglyceride (Z_{35}=1.79, P=0.86; Fig. 5B). OXY did not differ significantly between treatment groups (Z_{46}=0.70, P=0.48; Fig. 5D). However, HF treatment induced significantly higher dROMs (Z_{38}=−2.06, P=0.04, Cohen's d=0.60; Fig. 5E) than CTR treatment, even after controlling for OXY (Z_{38}=−2.11, P=0.03, Cohen's d=0.64).

DISCUSSION

We used the technique of Koetsier and Verhulst (2011) to experimentally manipulate foraging behaviour in zebra finches and investigate physiological correlates of 'exercise' (*sensu* Halsey, 2016) and increased foraging effort. Birds in the experimental 'high foraging cost' group (HF) dramatically changed their foraging behaviour upon removal of perches: they made repeated, short (30 cm) vertical flights from the cage bottom to the feeder, or hovered at the feeder, whereas controls obtained seeds by perching on the feeder for more prolonged periods. HF birds made significantly more trips to the feeder per unit time but spent less total time at the feeder than control birds. This is probably due to differences in foraging behaviour between the two treatment groups, as well as the way we scored foraging behaviour, where any time spent at or near the feeder was included. To illustrate the differences in foraging behaviour, HF birds had to hop to and hover briefly in

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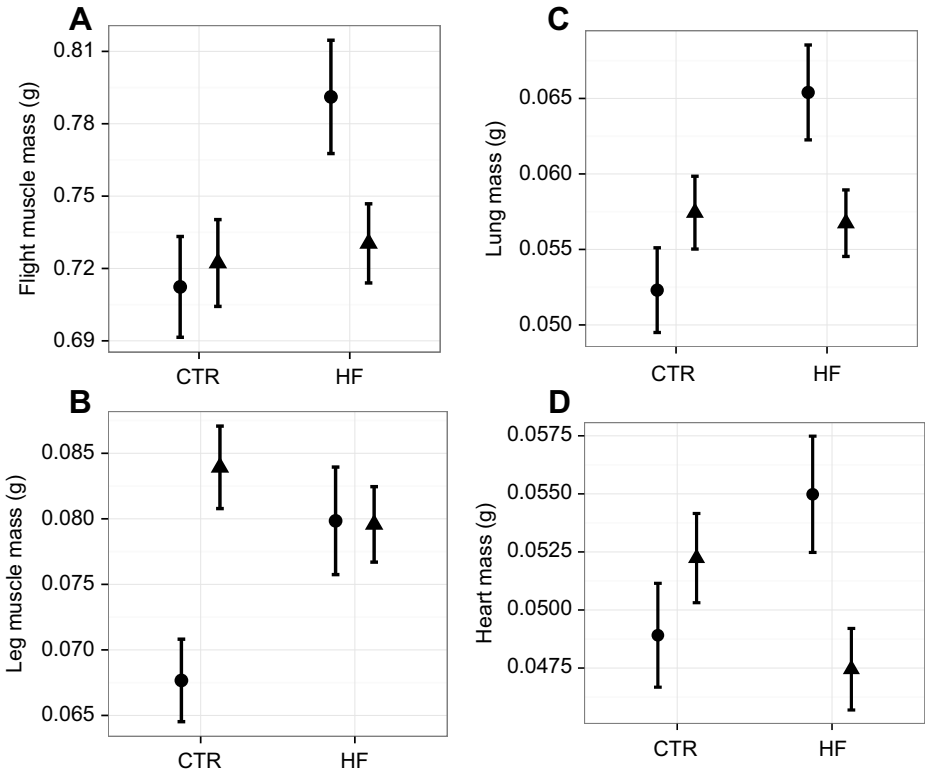

Fig. 4. Effects of foraging treatment on body composition. The high foraging cost treatment significantly increased (A) flight muscle mass, (B) leg muscle mass, (C) lung mass, and (D) heart mass in females (circles) but decreased heart mass in males (triangles). HF, high foraging group; CTR, control birds. Data shown are least-squared means±s.e.m.

front of the feeder placed ∼30 cm above the cage floor multiple times in order to obtain seeds, while control birds sat and perched on the feeder while they fed. This foraging mode in HF zebra finches mimics the energetically costly foraging typical of small free-living passerines (Tinbergen and Dietz, 1994). Furthermore, the effect of increased foraging effort on number of foraging trips to the feeder is comparable in magnitude to Koetsier and Verhulst (2011). Despite this marked change in foraging behaviour we documented few short- or long-term effects of 'training' or 'exercise', and some of these effects were sex specific. There was a transient decrease in body mass in HF birds immediately after removal of perches, but body mass recovered to pre-treatment levels subsequent to a short-term drop. This finding differs somewhat from the findings of Briga and Verhulst (2017), where birds subjected to high foraging cost weighed on average 4% less than control birds. There was no effect of foraging treatment on BMR, Hct, Hb or plasma glucose, glycerol and triglyceride levels. HF females had higher flight muscle, leg muscle and heart mass compared with controls, but HF males had lower heart mass than controls, and there was no effect of treatment on kidney and digestive organs. Finally, HF birds had a higher level of oxidative stress, with higher levels of reactive oxygen metabolites (dROMS) but similar anti-oxidant (OXY) levels. It should be noted that body composition measurements were carried out at a different time point relative to metabolic rate and physiology measurements. Therefore, the possibility of temporal variation in body composition in relation to training could not be ruled out.

Zebra finches in our high foraging cost treatment obtained seeds by making repeated, short (30 cm) vertical flights from the cage bottom to the feeder, or by hovering at the feeder. Tinbergen and Dietz (1994) showed that great tits (*Parus major*) spent less than 20% of their total time budget flying, while foraging for food to feed their chicks, yet their daily energy expenditure increased with body mass with an exponent of 1.99 (cf. exponent=0.657 for the interspecific relationship between DEE and mass; Daan et al., 1991). They suggested that the high energetic cost of small jumps and hovers was more mass dependent than longer, sustained flight, due to low flight costs and frequent accelerations. In captivity, zebra finches feed throughout daylight hours, with some diurnal variation. Foraging distance of our captive birds calculated using data collected from our behavioural observations yielded ∼0.65 km day^{-1}, within the range of foraging distance in free-living zebra finches (Mariette et al., 2011). Although we did not measure DEE in our study, we found no effect of treatment on BMR, contrary to the findings of Koetsier and Verhulst (2011) and Briga and Verhulst (2017). However, the possibility of undetected energy savings could not be ruled out because it has been found that experimental effects of increased foraging costs on metabolic rate were stronger with decreasing temperature (Briga and Verhulst, 2017). Mathot and Dingemanse (2015) suggested that BMR and DEE can be related to each other in different ways. The 'independent allocation model' proposed that the amount of energy available above basic maintenance costs is independent of maintenance metabolic rate (i.e. BMR), and hence individuals can increase DEE independent of BMR (Mathot and Dingemanse, 2015; Portugal et al., 2016). Furthermore, behavioural observations suggested that they did increase workload in response to HF treatment. Birds in the HF group were also found to have more seeds in their oesophagus at the time of tissue collection, suggesting that food intake was higher in HF birds. However, the possibility of a treatment effect on total food intake being an artefact of treatment effect on temporal food intake patterns (i.e. foraging bouts being more spread out throughout the day in HF birds) could not be ruled out.

Even though HF birds markedly changed their foraging behaviour they were apparently able to maintain food intake and energy balance as their body mass was not different from pre-treatment mass even after 90 days. In other studies that employed exercise training in birds, Costantini et al. (2012) and Briga and

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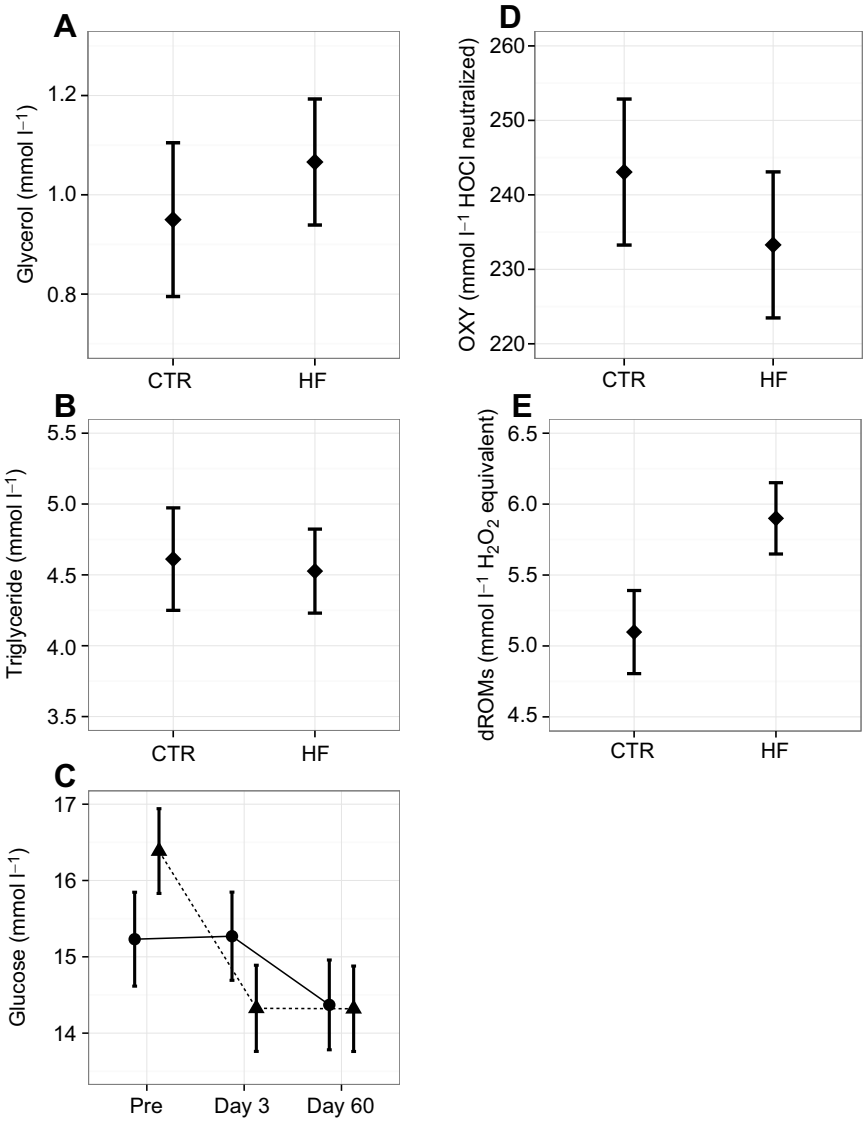

Fig. 5. Effects of foraging treatment/effort on plasma metabolites and oxidative stress. The high foraging cost treatment did not affect (A) glycerol, (B) triglyceride, (C) blood glucose and (D) total anti-oxidant capacity (OXY), but significantly increased (E) reactive oxygen metabolites production (dROMs). Filled circles and continuous lines represent control birds; filled triangles and dashed lines represent HF birds. Data shown are least-squared means±s.e.m.

Verhulst (2017) reported a decrease in body mass in exercise-trained birds, whereas Zhang et al. (2015) reported an increase in body mass in exercise-trained birds. Similar to Costantini et al. (2012), our study found that HF birds showed a slight (but not statistically significant) initial decrease in body mass at day 3 but then recovered to pre-treatment mass at day 60, but unlike Zhang et al. (2015) we did not detect a subsequent increase in body mass. Many other studies in birds also found either no change or a decrease in body mass when exposed to increased foraging cost (summarized in Wiersma and Verhulst, 2005). This discrepancy could be due to differences in training method (e.g. food availability) or length of training period (~60 days in our study versus 24 days in Zhang et al., 2015). Taken together, it appears that HF birds expended more energy and consumed more energy in response to increased foraging effort.

Despite evidence for higher instantaneous food intake in HF birds compared with controls (based on higher crop contents), we did not detect any changes in digestive organs in HF birds. To address the potential issue of low sample size and to obtain an indication of an upper limit of body composition effects that could have gone undetected, a *post hoc* power analysis was conducted with both sexes pooled. The analysis suggested that with 90% power, we could have established an effect size of 0.89, suggesting that undetected effects were smaller than 0.89. It should also be noted that our finding of higher instantaneous food intake in response to increased foraging cost contrasts with most studies that manipulated foraging effort in birds (summarized in Wiersma and Verhulst, 2005), but consistent with findings by Wiersma et al. (2005). However, for organs related to aerobic and metabolic capacity (i.e. exercise organs), male and female birds appeared to adopt different strategies in response to increased foraging costs. Whereas HF females upregulated a suite of exercise organs such as flight muscle, leg muscle, heart and lungs presumably to cope with the high workload, HF males decreased their heart mass and did not change other organs. A number of studies investigating the relationship between exercise and body composition in mammals and lizards suggested that exercise performance generally exhibits weak positive correlations with organ masses (Chappell et al., 2007) and that level of workload or endurance training usually elicits changes in body composition (Garland et al., 1987; Swallow et al., 2010), although the direction and magnitude of changes are rather inconsistent among taxa and specific studies, presumably due in part to training regime and food availability. Nevertheless, findings from these studies, together with studies on migratory birds

(Piersma, 1998; Guglielmo and Williams, 2003) suggested that birds that are trained to work harder should upregulate both exercise and digestive organs (Swallow et al., 2010) to cope with the increased workload. Increased workload corresponds to increased food consumption based on data from our study as well as studies in mice (e.g. Copes et al., 2015). Studies have also shown a positive correlation between food consumption and gut size (Mathot et al., 2017), presumably because a larger gut allows animals to eat more and be more efficient at processing food. Alternatively, birds exposed to increased workload could downregulate metabolically expensive organs as an energy-saving mechanism to avoid exceeding the 'metabolic ceiling' and face increases in mortality risk (Piersma, 2011). Similar to the physiological changes observed in migratory birds preparing for long-distance flight (Piersma, 1998; Guglielmo and Williams, 2003), HF females increased mass of organs associated with metabolic and aerobic capacity. In contrast, HF males decreased their heart mass, possibly as an energy-saving mechanism. This particular finding is consistent with other studies in mammals and lizards (Scheuer and Tipton, 1977; Garland et al., 1987), which also report a decrease in heart mass in response to endurance training. Furthermore, the sex-specific adjustments observed in our study could also be attributed to differences in wing length between male and female birds. Like many other passerine species, female zebra finches have shorter wings, and thus higher wing loading than male zebra finches (K.N.Y., unpublished data). Therefore, it is plausible that females have to upregulate mass of organs associated with metabolic and aerobic capacity in response to increased workload, as a means to compensate for the comparatively higher wing loading.

Birds in the HF treatment did not show any adjustments in other traits associated with aerobic capacity (Hct and Hb) compared with controls. This finding is inconsistent with the widely established positive correlation between energy expenditure or workload and Hct or Hb in interspecific studies (Fair et al., 2007; Lourdais et al., 2014), but consistent with the findings of some intraspecific studies, which found no effects of workload on Hct or Hb (Burness et al., 2001; Schumacher et al., 2002).

We found no evidence for adjustment of traits associated with fuel use or energy supply (glycerol, triglyceride, glucose) despite the observed changes in foraging behaviour. It should be noted that the order in which birds are being sampled was randomized, and hence approximately the same number of birds in each treatment group was sampled in the morning and in the afternoon. Studies on migratory birds exercising at high intensity for long durations indicated that they use predominantly lipids to fuel energetically demanding migratory flight (Piersma, 1990; Piersma and Jukema, 1990; Egeler and Williams, 2000). Glucose is known to be an important fuel for 'fast-twitch' muscle fibres responsible for a sudden burst of activity (Hultman, 1995; Weber and Haman, 2004; Melendez-Morales et al., 2009) such as take-off flight in birds. Given that foraging flight in most passerines often involves landing and take-offs interspersed between multiple sustained flights, we had expected that birds that forage more (i.e. HF trained birds) would have higher levels of triglyceride and glucose compared with controls. The lack of adjustment in lipid and glucose metabolism in HF birds is perhaps not unsurprising considering that birds exercise at lower intensity during foraging compared with migration (Piersma, 2011). Although there is evidence from studies of migratory birds suggesting that glucose level decreases in response to exercise (Hullár et al., 2008; Gerson and Guglielmo, 2013), we are not aware of any studies that investigated the effects of long-term endurance training on glucose in birds.

Although we found little evidence for physiological adjustments to support increased workload, we did find evidence that the high-foraging costs treatment generated a potential physiological cost. Although HF and control birds did not differ in their total anti-oxidant capacity, HF birds had higher plasma levels of reactive oxygen metabolites, which suggests that increased foraging cost causing increased oxidative stress (i.e. a cost of high workload) (Stier et al., 2012), consistent with other studies showing a link between high levels of ROS production and exercise (Costantini et al., 2008; Alan and McWilliams, 2013; Jenni-Eiermann et al., 2014). Taken together, these findings indicate that working hard does perhaps come at a cost in the form of increased oxidative stress.

In summary, our study has shown that despite the significant behavioural adjustment observed in birds that were made to 'work harder', surprisingly few physiological adjustments were observed, especially in the case of male birds. However, given the relationship between increased workload and increased oxidative stress, an obvious next step is to investigate fitness consequences of high foraging costs. Briga et al. (2017) found that birds reared in harsh environmental conditions had a shorter lifespan when subjected to increased foraging cost. Simons et al. (2014) found that increased foraging cost during reproduction can negatively affect breeding success. However, the physiological link between increased foraging effort and reduced reproductive fitness has not been established. We know that the physiological costs of activity can often be deferred from one life-history stage to a later stage, i.e. there can be carry-over effects (Harrison et al., 2011; Williams and Fowler, 2015). Future studies could repeat the training protocol described above and investigate the link between training, physiology and reproduction. Whether the higher oxidative stress caused by increased foraging costs would reduce reproductive success remains to be determined.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.N.Y., T.D.W.; Methodology: K.N.Y., T.D.W.; Validation: K.N.Y., O.R.K.; Formal analysis: K.N.Y., K.C.H.; Investigation: K.N.Y., O.R.K., K.C.H.; Resources: K.N.Y., T.D.W.; Data curation: K.N.Y.; Writing - original draft: K.N.Y.; Writing - review & editing: K.N.Y., T.D.W.; Visualization: K.N.Y.; Supervision: T.D.W.; Project administration: K.N.Y., T.D.W.; Funding acquisition: T.D.W.

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Data availability
Data have been deposited in the Dryad Digital Repository (Yap et al., 2017): https://doi.org/10.5061/dryad.7qs70

Supplementary information
Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.160812.supplemental

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Fig. S1. Picture of high foraging cost treatment (HF) cage setup.

Fig S2. Plot of all tissue masses in (A) females and (B) males. Filled circles represent CTR birds; Filled triangles represent HF birds. “lint” = large intestine; “sint” = small intestine”; “pect”=flight muscle. Data shown are least-squared means ± s.e.

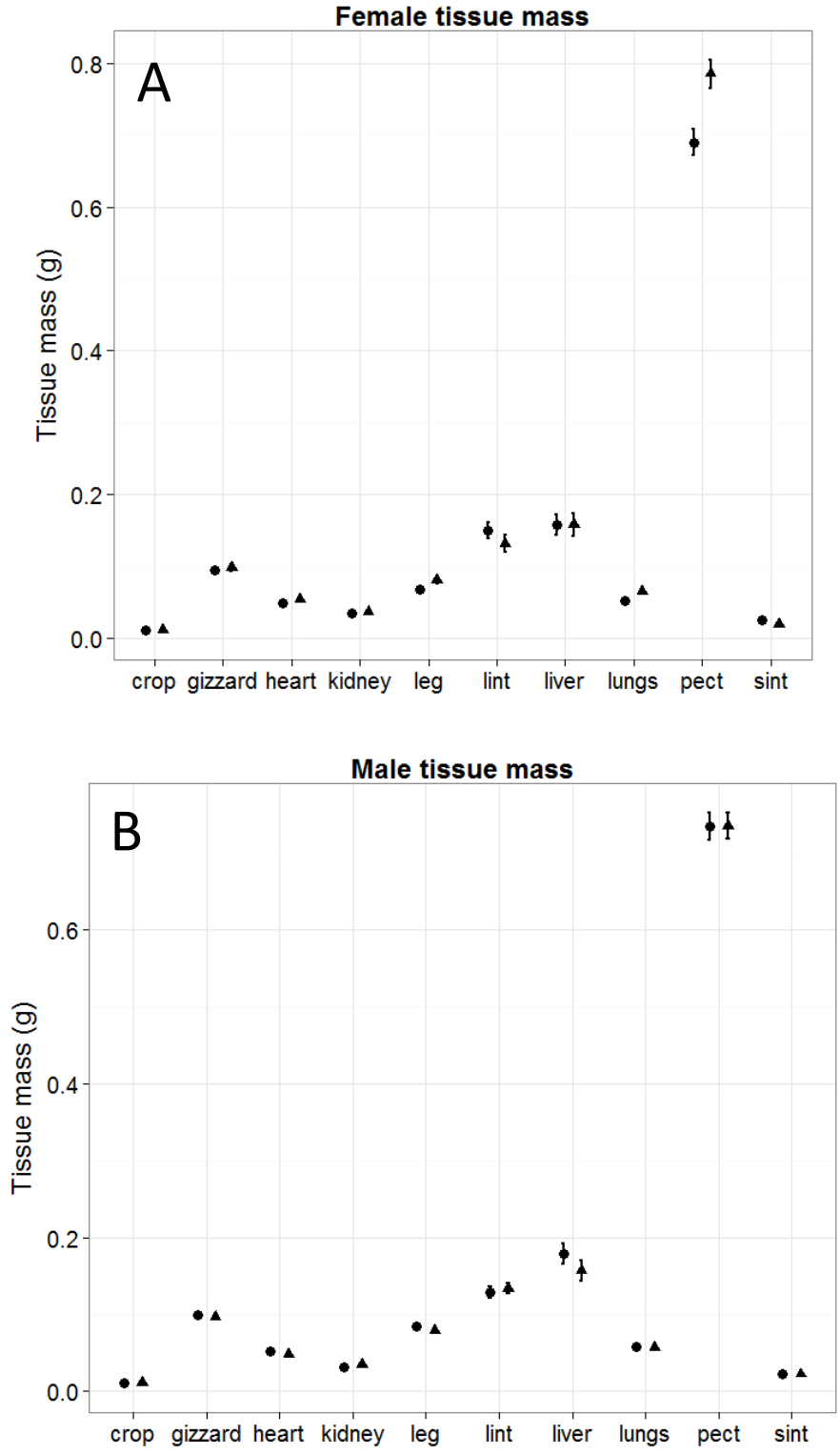

Fig S3. Day 3 and Day 60 data for (A) body mass, (B) basal metabolic rate (BMR), (C) hematocrit (Hct) and (D) hemoglobin (Hb), using pre-treatment (Day 0) values as covariate. Filled circles and solid lines represent CTR birds; Filled triangles and dashed lines represent HF birds. Data shown are least-squared means ± s.e.

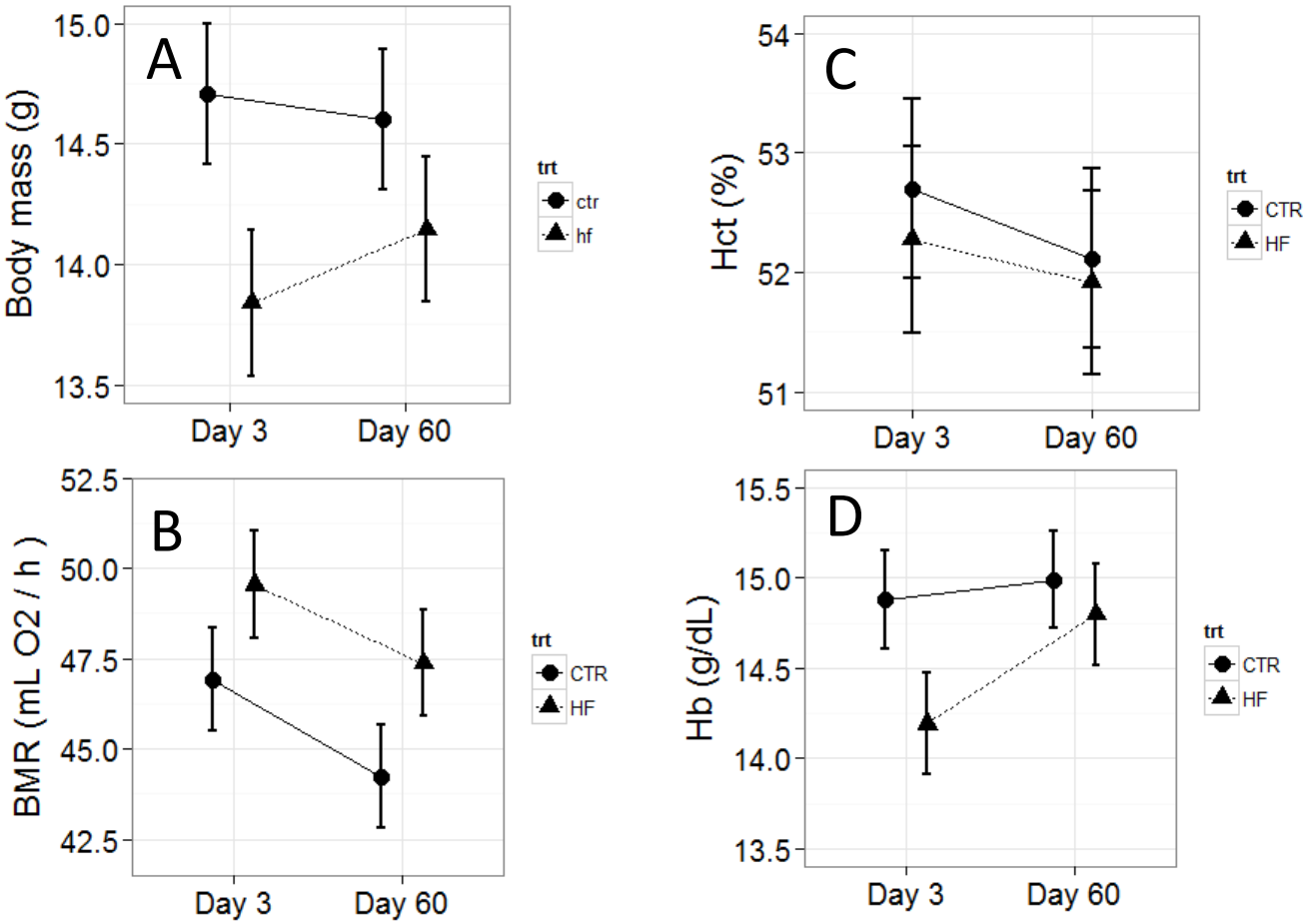

Table S1. Sample sizes for each physiological measurement (organized by sex and treatment)

	HF Male	HF Female	CTR Male	CTR Female
BMR	18	9	18	11
Hct	18	9	18	11
Hb	18	9	18	11
Body composition	18	9	16	11
Glucose	10	6	8	5
Triglyceride and glycerol	15	7	7	8
OXY	16	8	13	11
dROMs	15	8	9	8

Table S2. Statistical model showing Time by Treatment interaction in body mass, BMR, hematocrit, hemoglobin, and glucose, and treatment effect on behavior, immediate food consumption, tissue masses, glycerol, triglyceride, OXY and dROMs. Data shown are least-squared means ± s.e. with both sexes pooled.

Trait	Pre-trt		Day 3		Day 60		Day 90		Random factor	Estimated Variance	Residual Variance	numDF	denDF	*W*-value	*Z*-value	*F*-value	*P*-value
	CTR	HF	CTR	HF	CTR	HF	CTR	HF									
Body mass (g)	14.43 ± 0.29	14.37 ± 0.30	14.69 ± 0.29	13.85 ± 0.30	14.60 ± 0.29	14.15 ± 0.30	NA	NA	Bird ID	1.930	0.468	2	108	.	.	4.499	0.01
BMR (mL O2/h)	44.72 ± 1.32	48.42 ± 1.37	47.08 ± 1.32	49.47 ± 1.38	44.39 ± 1.32	47.35 ± 1.37	NA	NA	Bird ID	7.491	42.834	2	107	.	.	0.137	0.87
Hematocrit (%)	52.69 ± 0.72	51.30 ± 0.74	52.67 ± 0.72	53.62 ± 0.75	52.05 ± 0.72	51.96 ± 0.75	NA	NA	Bird ID	9.479	5.500	2	107	.	.	1.163	0.32
Hemoglobin (g/dL)	14.91 ± 0.26	14.35 ± 0.27	14.82 ± 0.27	14.23 ± 0.28	14.92 ± 0.26	14.86 ± 0.27	NA	NA	Bird ID	0.937	1.079	2	107	.	.	1.095	0.34
Glucose (mmol/L)	15.23 ± 0.62	16.39 ± 0.56	15.27 ± 0.58	14.33 ± 0.56	14.37 ± 0.59	14.32 ± 0.56	NA	NA	Bird ID	1.393	3.578	2	53	.	.	2.225	0.12
Trips to feeder	NA	NA	NA	NA	NA	NA	1.921 ± 2.130	15.843 ± 2.209	.	.	.	1	54	215	.	.	< 0.01
Time spent resting (s)	NA	NA	NA	NA	NA	NA	1106.66 ± 61.831	1011.625 ± 59.382	.	.	.	1	54	.	1.10	.	0.27
Immediate food consumption (g)	NA	NA	NA	NA	NA	NA	0.177 ± 0.029	0.275 ± 0.029	.	.	.	1	54	215	.	.	0.009
Leg muscle mass (g)	NA	NA	NA	NA	NA	NA	0.077 ± 0.003	0.080 ± 0.003	.	.	.	1	52	.	-0.79	.	0.43
Flight muscle mass (g)	NA	NA	NA	NA	NA	NA	0.719 ± 0.014	0.750 ± 0.014	.	.	.	1	52	.	-1.59	.	0.11
Heart mass (g)	NA	NA	NA	NA	NA	NA	0.509 ± 0.001	0.500 ± 0.001	.	.	.	1	52	.	0.41	.	0.68
Lung mass (g)	NA	NA	NA	NA	NA	NA	0.055 ± 0.018	0.061 ± 0.019	.	.	.	1	52	.	-1.59	.	0.11
Crop mass (g)	NA	NA	NA	NA	NA	NA	0.012 ± 0.0008	0.011 ± 0.0008	.	.	.	1	52	.	0.54	.	0.59
S. intestine mass (g)	NA	NA	NA	NA	NA	NA	0.023 ± 0.001	0.021 ± 0.001	.	.	.	1	52	.	1.09	.	0.27
L. intestine mass (g)	NA	NA	NA	NA	NA	NA	0.138 ± 0.007	0.132 ± 0.007	.	.	.	1	52	.	0.59	.	0.56
Gizzard mass (g)	NA	NA	NA	NA	NA	NA	0.097 ± 0.003	0.096 ± 0.003	.	.	.	1	52	.	-0.10	.	0.92
Liver mass (g)	NA	NA	NA	NA	NA	NA	0.171 ± 0.01	0.157 ± 0.01	.	.	.	1	52	.	1.01	.	0.31
Kidney mass (g)	NA	NA	NA	NA	NA	NA	0.033 ± 0.002	0.035 ± 0.002	.	.	.	1	52	.	-0.73	.	0.47
Glycerol (mmol/L)	NA	NA	NA	NA	NA	NA	0.950 ± 0.155	1.067 ± 0.127	.	.	.	1	35	.	-0.57	.	0.57
Triglyceride (mmol/L)	NA	NA	NA	NA	NA	NA	4.611 ± 0.362	4.527 ± 0.296	.	.	.	1	35	.	0.18	.	0.86

OXY (mmol/L HOCl neutralized)	NA	NA	NA	NA	NA	NA	243.06 ± 9.803	233.28 ± 9.803	.	.	.	1	46	.	0.70	.	0.48
dROMs (mmol/L H2O2 equivalent)	NA	NA	NA	NA	NA	NA	4.919 ± 0.318	5.769 ± 0.253	.	.	.	1	38	.	-2.11	.	0.03