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RESEARCH ARTICLE

Unpredictable food availability induces metabolic and hormonal changes independent of food intake in a sedentary songbird

H. Bobby Fokidis[1,2,*], Matthieu Burin des Roziers[3], Richard Sparr[4], Christopher Rogowski[2], Karen Sweazea[5] and Pierre Deviche[2]

[1]Department of Psychology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada, [2]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA, [3]Universite de Poitiers, U.F.R. Sciences Fondamentales et Appliquees, 86022 Poitiers Cedex, France, [4]Department of Applied Science and Mathematics, Arizona State University, Mesa, AZ 85212, USA and [5]College of Nursing and Health Innovation, Arizona State University, Phoenix, AZ 85004, USA

*Author for correspondence (hbfokidis@psych.ubc.ca)

SUMMARY

Environments often vary with regard to their temporal resource availability, but little is understood concerning how resource predictability impacts animals. The adaptive regulation hypothesis suggests that organisms act to conserve their current energetic state during periods of diminished food access and recuperate their energetic reserves (fat and muscle) during periods of greater food availability. In contrast, the chronic stress hypothesis suggests that variation in access to food can induce a prolonged stress response, resulting in maladaptive usage of energy reserves and increased behavioral activity. To distinguish between these hypotheses we compared the behavioral, hormonal and metabolic responses of captive curve-billed thrashers, *Toxostoma curvirostre*, fed varying amounts each day (variable group) with those of birds fed a constant amount every day (constant feeding group). Birds of both groups consumed, on average, a similar total amount of food during the course of the study, but birds in the variable feeding group lost mass and increased their circulating initial levels of the stress hormone corticosterone, showed evidence for increased secretion of a hypothalamic stress peptide, vasotocin, used greater amounts of fat and protein energy reserves, and were more behaviorally active than birds in the constant feeding group. Overall, these findings support the chronic stress hypothesis and suggest that birds such as thrashers may be particularly susceptible to the perception of unpredictable variation in food supplies independent of actual energetic constraints.

Key words: chronic stress, food variability, predictability, curve-billed thrasher, corticosterone, energy reserve, vasotocin, fault bar, gluconeogenesis, lipid, protein, glucose.

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INTRODUCTION

The amount of available energy (i.e. food quantity and quality) is a primary limiting factor influencing many physiological processes including reproduction (Fernandez-Fernandez et al., 2006; dos Santos et al., 2010), growth (Cox et al., 2008; Fan et al., 2008), development (Corbel et al., 2010) and the immune system (McGraw et al., 2006; Houston et al., 2007). Energetic status may also act as a proximate cue for timing reproductive activities in seasonal breeders (Hau et al., 2000; Schoech and Hahn, 2005). The relationship between energy and physiology is exemplified by the numerous energy storage mechanisms found in vertebrates (Arrington et al., 2006; Naya et al., 2008; Smith and McWilliams, 2010). Fat reserves, in the form of triglycerides, enable organisms to maintain the metabolic demands of self-maintenance and activity associated with periods of food scarcity (Rogers and Reed, 2003; Rozman et al., 2003). Prolonged periods of food scarcity can lead to loss of body mass due to the depletion of fat (breakdown to free glycerol) and protein (breakdown to amino acids) reserves for fueling gluconeogenesis (Fery et al., 1996; Wingfield, 2003). This depletion concurs with allocation of energy towards vital 'life-sustaining' physiological functions (Wingfield, 2003). In situations where food scarcity is followed by abundance, many organisms engage in 'compensatory hyperphagia' that results in replenishment of energy reserves (Bull and Metcalfe, 1997; Jobling and Johansen, 1999). The effects of food quantity and quality on physiology have been well researched (Brown and Sherry, 2006; Houston et al., 2007; Martinez-Padilla and Fargallo, 2007; Jenni-Eiermann et al., 2008), but less is known regarding how temporal variability in these resources can influence physiology and, particularly, the depletion of energy reserves. Further complication arises because food variability may constitute a significant stressor that can also alter metabolism.

The physiological response to stress involves the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Hazard et al., 2007). This activation causes the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in mammals [or arginine vasotocin (AVT) in other vertebrates (Antoni, 1993; Hazard et al., 2007)] from the hypothalamus, adrenocorticotropin hormone (ACTH) from the pituitary gland, and glucocorticoids [cortisol or corticosterone (CORT)] from the adrenal glands (Hazard et al., 2007). A primary consequence of HPA activation is hyperglycemia (i.e. elevation of blood glucose level) *via* gluconeogenesis, which depletes energy reserves (Dallman et al., 2007). Food restriction can elevate glucocorticoid levels (Díaz-Muñoz et al., 2000; Lynn et al., 2003) and this increase is associated with a decline of non-vital processes such as reproduction and immunity (French et al., 2007; French and Moore, 2008). In addition, CORT increases daily foraging activities (Astheimer et

al., 1992; Lohmus et al., 2006) and can promote locomotor activity in captivity (Lynn et al., 2003; Fokidis et al., 2011a). These behavioral changes can also alter the energy balance. By responding to changes of the current energetic state, the HPA axis plays an important role in mediating transitions from regular 'life history' activities to vital 'emergency' functioning.

In environments, such as those subjected to frequent inclement weather and where food availability can rapidly vary temporally, the relationship between maintenance of energy reserves and the stress of 'unpredictable' foraging has been considered in a risk foraging framework (Bednekoff and Houston, 1994). This framework suggests that periods of variable food intake due to variation in food abundance or interruptions in foraging due to increased 'risk' (such as predation or inclement weather) prompt individuals to regulate their body mass and energy usage (Bednekoff and Krebs, 1995; Witter et al., 1995; Cuthill et al., 2000). In birds and mammals, temporal variability in food availability is associated with fattening (Bednekoff and Krebs, 1995), an increase in daily torpor (Munn et al., 2010), increased or stabilized body mass (Witter et al., 1995; Cuthill et al., 2000) and less behavioral activity (Dall and Witter, 1998). These observations are consistent with the adaptive regulation hypothesis (Witter et al., 1995; Fauchald et al., 2004), which describes how animals coping with unpredictable food availability aim to decrease energetic expenditure and attenuate physiology that depletes energy reserves such as CORT secretion, thus promoting energy conservation, which conserves (or even increases) body mass.

Although this hypothesis describes an adaptive process, other studies have demonstrated that variable access to food can decrease body mass (Acquarone et al., 2002; Cucco et al., 2002) and growth rate (Boon et al., 1999), and lower plasma testosterone (Bridge et al., 2009). These observations suggest that energy is being mobilized and contradict the adaptive regulation hypothesis. One potential mediator of such responses is CORT released during chronic stress, which acts to depreciate body condition and mobilize intrinsic energy reserves. According to this chronic stress hypothesis, elevated glucocorticoid levels may prolong their catabolic actions and interfere with energy conservation. Thus the chronic stress hypothesis characterized by increased CORT secretion, depreciated body mass and greater energy expenditure would be maladaptive and is likely to be unsustainable under natural conditions of food unpredictability.

Birds serve as useful models for addressing the relationships between CORT and energy. Birds have higher mass-specific metabolic rates, blood glucose levels, glucose tolerance and insulin resistance than other vertebrates (Braun and Sweazea, 2008), and yet are capable of living longer than mammals of similar size despite their apparently higher energetic demand (Holmes and Ottinger, 2003). To meet their elevated energy demand during periods of nutritional stress, birds utilize fat and protein stores faster than mammals (Morton et al., 1994; McWilliams et al., 2004) and they are consequently also more responsive than mammals to experimental food manipulations. Most previous research on the impact of food variability focused on bird species with large fat reserves for enduring temperate winter overnights (Bednekoff and Krebs, 1995; Witter and Swaddle, 1997; Pravosudov et al., 2001; Polo and Bautista, 2002). Other studies investigated birds known to hoard food during periods of food shortage (Hurly, 1992; Acquarone et al., 2002; Cucco et al., 2002; Bridge et al., 2009). To our knowledge, only two studies have attempted to relate the temporal availability of food to HPA activity. Bridge et al. (Bridge et al., 2009) reported no difference in CORT secretion between western scrub jays (*Aphelocoma californica*) subjected to 'unpredictable' and 'predictable' feeding regimes. In another study on European starlings (*Sturnus vulgaris*), an intermittent food restriction lasting 20 days resulted in attenuated CORT secretion during the food restriction period, but only in molting birds (Bauer et al., 2011). However, starlings in the above study did not exhibit signs of chronic stress after they were allowed to recover from the food restriction, suggesting that they are fairly resistant to intermittent fasting. These above studies differed with respect to the methodology used to control food access. They involved manipulations of: (1) the time available to forage (Witter et al., 1995; Boon et al., 1999); (2) the timing of food presentation (Boon et al., 1999); (3) the amount of food provided, but not necessarily consumed (Acquarone et al., 2002; Cucco et al., 2002; Munn et al., 2010); (4) the rate of food consumption, but not the absolute amount consumed (Bridge et al., 2009); or (5) the interruptions to foraging (Dall and Witter, 1998; Bauer et al., 2011). In addition, many of these studies used control groups with access to *ad libitum* food as the basis for comparisons, but did not determine the actual amount of food consumed. As a result, they did not separate the effects of perception of variable food access from those resulting from actual energy intake.

To address this issue, we manipulated the amount of food consumed daily by captive curve-billed thrashers, *Toxostoma curvirostre* (Swainson 1827), a primarily insectivorous species that inhabits xeric environments of the Sonoran Desert (Tweit, 1996). As year-round residents of this habitat, thrashers lack the elaborate fat storage mechanisms found in migratory species and show little furcular fat deposition at any time of the year, although they have substantial amounts of muscle that can be used for energy (H.B.F., personal observation). The primary food source of thrashers (insects) is susceptible to deteriorating environmental conditions such as colder temperatures and inclement weather. Experimental evidence demonstrated a strong relationship between current body mass (and condition) and CORT secretion with food restriction. Specifically, food-restricted birds had increased initial ('pre-stress') CORT in plasma and a decreased capacity to increase CORT levels after handling stress (Fokidis et al., 2011a). This observation is consistent with field studies on the same species. In these studies, birds inhabiting urban areas were heavier and had a more robust CORT response to handling and restraint stress than birds living in native desert habitats (Fokidis et al., 2009; Fokidis and Deviche, 2011). For the present study, we measured parameters related to the usage of energy reserves, the activity of the HPA axis and behavioral responses to food variability in birds whose daily but not overall (i.e. over the course of several days) amount of food consumed was experimentally manipulated. The adaptive regulation hypothesis predicts that birds will constrain metabolic, hormonal and behavioral functions that deplete energy reserves (fat stores, muscle) in order to minimize the loss of body mass (Table 1). In contrast, the chronic stress hypothesis predicts that increased activity of the HPA axis will deplete energy reserves and promote energetic expenditure (i.e. increased activity) resulting in loss of body mass and deteriorating body condition (Table 1).

MATERIALS AND METHODS

All procedures in this study followed guidelines approved by the Arizona State University Institutional Animal Care and Use Committee and were performed under scientific collecting permits from the US Fish and Wildlife Service, US Forest Service, and Arizona Game and Fish Department, and with permission from local landowners.

Table 1. Predicted responses of physiological and behavioral variables in captive curve-billed thrashers, *Toxostoma curvirostre*, to an unpredictable daily food supply

Measure	Adaptive regulation	Chronic stress
Morphometric		
Body mass	↑ or –	↓
Pectoralis muscle mass	↑ or –	↓
Liver mass	↑ or –	↓
Gizzard mass	↑ or –	↓
Spleen mass	↓	↑ or –
Metabolic		
Osmolality	–	↓
Plasma triglycerides	↑	↓
Plasma free glycerol	↓	↑
Total plasma protein	↓	↑
Hormonal		
Initial plasma CORT	↓	↑
Stress plasma CORT	↓	↑
H/L ratio	↑	↓
Pvn staining intensity	↓	↑
No. of AVT-ir cells	↓	↑
AVT-ir within cells	↓	↑
ME staining intensity	↓	↑
Behavioral		
Time spent perched	↑	↓
No. of hops	↓	↑

The adaptive regulation hypothesis predicts energy conservation in the face of an unpredictable food supply. The chronic stress hypothesis predicts a deterioration of energetic state with unpredictability in food supply. ↑ and ↓ indicate increases and decreases in the response of the variable, respectively, and – indicates no change.

Field sampling and housing

Adult male curve-billed thrashers (N=27) were captured at two locations: an unpopulated area (Four Peaks Mountains Wilderness) in the Tonto National Forest (AZ, USA; desert location; N=14), and urban areas in east-central Phoenix and Tempe, AZ (urban location; N=13). Desert and urban thrashers differ with respect to their body condition and stress physiology (Fokidis et al., 2009; Fokidis and Deviche, 2011; Fokidis et al., 2011b), but these differences disappear during captivity (Fokidis et al., 2011a).

Thrashers were lured to mist nets using conspecific song playbacks. The sex of each individual was assessed in the hand by the presence of a developed cloacal protuberance (CP), which in males is associated with breeding condition. Birds were captured between 05:30 and 09:57 h (mean capture time: urban, 07:36 h; desert, 07:08 h) and between 29 March and 23 June 2009 (mean capture date: urban, 5 June 2009; desert, 11 June 2009). This period coincides with the incubation and nestling stages of the reproductive cycle of the species (Tweit, 1996).

We brought birds into captivity at the Arizona State University Animal Care Facility, housed them in individual 76×46×46 cm metal cages located in the same room at 22°C, and exposed them to a photoperiod of 14 h of light followed with 10 h of darkness. Food consisted of a Mazuri insectivore diet (5MM3; PMI Nutrition, St Louis, MO, USA) containing 28% protein, 11% fat, 13% fiber and 8% ash. To determine the individual daily food intake (DFI), we calculated the difference between the amount of food provided to each bird at lights on (07:00 h), before birds began to feed that day, and the amount of food left 24 h later. Food containers were partly covered with cardboard so as to provide only a small opening for accessing food, which minimized food spillage. The DFI of each bird was measured for 45 consecutive days. All birds were weighed weekly.

Variable *versus* constant feeding treatments

Based on individual DFI values (mean DFI=11.7 g, range=10.6–12.4 g), we divided birds into a constant (control) and a variable (treatment) feeding group. Birds in the constant group received a daily amount of food equal to their DFI. By contrast, birds in the variable group received a daily amount of food that varied day to day between 30 and 200% of their respective DFI amount by mass. The amount of food provided to birds in the variable group was determined at random, but for each bird the specific amount of food given was corrected to equal the total amount of food provided to the constant group. Thus, over the course of the study, birds in both groups received an overall equivalent amount of food. All leftover food (mean=1.3 g, range=0.2–1.9 g) was measured each following day just after the lights came on to determine the amount of food consumed. The first trial was conducted for 18 days using 13 (N=7 desert, 6 urban) and 14 (N=7 desert, 7 urban) birds in the variable and constant feeding groups, respectively. At the completion of the study, the birds were provided access to food *ad libitum* for 15 days, during which time body mass was monitored weekly and allowed to recover to pre-treatment levels. The study was then repeated, but with each bird switched to the other experimental group (i.e. 14 and 13 birds in variable and constant feeding groups, respectively).

On the first day of each study trial (day 1), an (initial) blood sample (300 µl) was collected from the right jugular vein of each bird within 3 min of removal from the home cage using a heparinized 0.3 cc syringe with a 29.5 gauge needle. Birds were then held in a cloth bag for 30 min, after which a second ('stress') blood sample (200 µl) was collected. This protocol is widely used to induce an acute stress response (Wingfield et al., 1992; Fokidis et al., 2009; Fokidis et al., 2011b), defined as a rapid (within minutes) increase in plasma CORT concentration. Blood samples were also taken at days 9 (mid-point) and 18 (final) to determine changes in response to the experimental treatment. On days 8 and 17, all birds were allowed to feed at the DFI rate so that blood sampling on days 9 and 18, respectively, reflected the treatment integrated over more than only a single day. All blood samples were taken just after lights on and before birds had an opportunity to feed. Upon capture, birds were transferred to another room where blood and other data were collected to minimize disturbance to the colony. Birds were sampled at random with respect to origin or treatment, and time of sampling was not correlated with any variable measured (all $r \leq 0.16$, $P > 0.08$). Blood samples were stored on ice until plasma was separated by centrifugation, and then stored at –80°C until the various assays were run.

Monitoring behavioral activity

To estimate behavioral activity (as a proxy for energy expenditure) we concurrently video-recorded birds of both feeding groups (N=4 birds of each group per day) for 2 h each day (11:00–13:00 h). Each bird was video-recorded three times for a total of 6 h per bird. These recording times were chosen because they coincided with a period of time after the initial feeding for the day. Digital video recordings were then examined and two variables were measured: (1) the number of hops per minute, here defined as either hops from perch to perch, or perch to the cage floor, or *vice versa*; and (2) the time spent perching, defined as sitting perched inactive and without preening.

Inducing feather replacement and fault bar analysis

To determine whether variability in access to food influences feather growth, we induced feather replacement in all birds 5 days after the

termination of the first trial, meaning birds re-grew feathers during the course of the second trial. A left retrix (tail) [third from the right=R4 (Pyle, 1997)] was removed and allowed to start growing 10 days prior to the start of the second trial. At the conclusion of the study, after birds were euthanized (see below), the re-grown tail feathers were removed and measured (from tip to base) and the number of fault bars on each feather was manually counted. Fault bars are obvious translucent bands across the feathers resulting from stressful conditions that impact barbule formation (Prentice et al., 2008). Fault bars often coincide with points of feather breakage and thus are viewed as a handicap (Serrano and Jovani, 2005; Prentice et al., 2008).

Plasma corticosterone

Total plasma CORT concentrations were measured using commercial competitive ELISAs (Assay Designs, Ann Arbor, MI, USA) as previously described and validated in Fokidis et al. (Fokidis et al., 2009). All samples from the same individual were run on the same assay plate. The sensitivity of the CORT assay ranged from 9.9 to 15.1 pg ml^{-1} depending on the plate, and the mean intra-assay coefficient of variation was 16.2% (N=5 plates; 162 samples assayed in duplicate).

Triglycerides, glycerol and glucose

Plasma free glycerol and triglycerides were measured using a sequential color endpoint assay (reagents F6428 and T2449, Sigma-Aldrich, St Louis, MO, USA) (for details, see Guglielmo et al., 2002; Guglielmo et al., 2005; Fokidis et al., 2010; Fokidis et al., 2011a). The 'true' triglyceride concentration was defined as the difference between the total triglycerides (i.e. triglycerides and free glycerol) and the free glycerol concentrations. Plasma glucose was also measured using a commercial enzyme endpoint assay (#10009582, Cayman Chemical Co., Ann Arbor, MI, USA). Metabolite concentrations are expressed in mmol l^{-1} to facilitate comparisons. Assay sensitivities and mean intra- and inter-assay coefficients of variation are as follows: free glycerol, 0.06–6.4 mmol l^{-1}, 6.7 and 14.0%; triglycerides, 0.09–15.1 mmol l^{-1}, 8.1 and 11.3%; and glucose, 2.5–27.01 mmol l^{-1}, 2.9 and 13.1%.

Plasma osmolality and total protein

Plasma osmolality (mOsm l^{-1} plasma), defined as the concentration of plasma solutes, was measured using a vapor pressure osmometer (model 5500XR, Wescor, Logan, UT, USA) with 50 µl samples assayed in duplicate. The osmometer was calibrated to known concentration standards before use (Fokidis et al., 2010).

Total plasma protein concentration was determined using a hand-held clinical refractometer with temperature compensation (model RHC-200, Huake Instrument Co., Shenzhen, China). A 20 µl drop of plasma was placed on the refractometer surface and the specific gravity was read according to the manufacturer's instructions. This provides a measure of the degree to which light passing through the sample is 'bent' or refracted by the presence of protein solutes (Haller, 2003). Before each reading, the refractometer was calibrated using distilled water, which has a specific gravity of 1.0. Previous research in a wide range of domestic and wild avian species has demonstrated that this technique provides a reliable and accurate measurement of total plasma proteins and that results are comparable to those obtained using biochemical methods of measurement (Dawson and Bortolotti, 1997; Cray et al., 2008; dos Santos Schmidt et al., 2008).

Heterophil to lymphocyte ratio

From the initial blood sample, approximately 5 µl was used to make a thin blood smear on a glass microscope slide for measuring the ratio of heterophil to lymphocytes (H/L ratio). Smears were air-dried at ambient temperature and stored until fixation. They were then fixed for 10 min in absolute methanol within 3 days of collection and subsequently Giemsa-stained (Bennett, 1970). Stained smears were then dehydrated for 1 week under partial vacuum. Stained slides were cleared using xylene, cover-slipped and sealed using Cytoseal 60 (VWR, San Francisco, CA, USA).

Chronic elevations in plasma CORT can suppress lymphocyte numbers, thereby increasing the H/L ratio (Harmon, 1998), and thus the H/L ratio has been used as a marker of stress (Gross and Siegel, 1983; Vleck et al., 2000). The number of heterophils and lymphocytes was counted under 400× magnification using an Olympus BX60 light microscope (Olympus Optical Co., Tokyo, Japan) until a total of 100 cells (both types combined) was reached (Fokidis et al., 2008; French et al., 2008). Cell types were identified using the criteria of Campbell (Campbell, 1996) and all slides were examined by a single observer (C.R.) without knowledge of individual, feeding group, locality or date of collection.

Organ masses and AVT immunohistochemistry

At the conclusion of the second study trial (day 18), birds were weighed, deeply anesthetized using metofane (methoxyflurane; Mallinckrodt, Mundelein, IL, USA) inhalation and euthanized by decapitation. Four birds were used for a separate study, and thus 12 and 11 birds from the variable and constant groups, respectively, were killed for this study. Brains were removed from the skull (3–6 min post-capture) and placed into 5% acrolein solution in 0.1 mol l^{-1} phosphate buffer (PB) overnight at 4°C (King et al., 1983; Luquin et al., 2010). In addition, the left and right pectoralis muscles, liver, spleen and gizzards were dissected out and weighed to the nearest 0.01 g. Brains were post-fixed and gelatin-embedded following a modified protocol outlined in Saldanha et al. (Saldanha et al., 1994) and Fokidis and Deviche (Fokidis and Deviche, 2012). Briefly, brains were rinsed three times with 0.1 mol l^{-1} PB (30 min each), immersed in 4% gelatin solution for 30 min and embedded in an 8% gelatin solution-filled mold. Gelatin was allowed to solidify overnight at 4°C. Gelatin-embedded brains were post-fixed in 4% paraformaldehyde for 48 h and then immersed in 10, 20 and 30% sucrose solutions for 48 h each. Brains were frozen on dry ice and stored at –80°C until sectioned.

Brains were coronally sectioned at 30 µm and every third section (i.e. at least 60 µm apart) was collected into cryoprotectant solution (Watson et al., 1986). Free-floating sections were stained for AVT using an indirect immunohistochemistry procedure (for details, see Fokidis and Deviche, 2012). Sections were washed three times with 0.1 mol l^{-1} PB for 30 min, incubated with 0.36% H_2O_2 for 15 min, washed three times with 0.1 mol l^{-1} PB (5 min each), incubated with normal horse serum [1:30 in PBT (PB with 0.3% Sigma Triton X-100); Sigma-Aldrich] for 1 h and then incubated overnight in 0.3% PBT containing anti-AVT polyclonal antibody (1:15,000; raised in rabbit and generously provided by Dr M. S. Grober, Georgia State University, Atlanta, GA, USA). The specificity of the AVT antibody has been previously validated in this species and is described in Fokidis and Deviche (Fokidis and Deviche, 2012). Sections were washed five times (10 min each) in 0.1 mol l^{-1} PB, incubated for 1 h in 1:100 biotinylated horse anti-rabbit IgG in 0.3% PBT (Vector Laboratories, Burlingame, CA, USA), washed three times for 10 min each in 0.1 mol l^{-1} PB, incubated in Vectastain Avidin-Biotin-Chromagen (ABC) solution (Vector Laboratories) for 1 h, washed

three times (15 min each) in 0.1 mol l^{-1} PB, and then incubated for 3 min in Vector SG peroxidase chromagen (Vector Laboratories) and washed twice for 5 min in 0.1 mol l^{-1} PB. Immunolabeled sections were mounted onto gelatin-coated glass microscope slides, air-dried at room temperature for 24 h, dehydrated with ethanol, cleared in xylene and coverslipped using Cytoseal 60 (VWR).

The production of AVT during stress occurs primarily within the paraventricular nucleus (PVN) of the hypothalamus, which releases AVT into the median eminence (ME) to eventually act on the pituitary gland. The distribution of AVT-like immunoreactivity (AVT-ir) in the midbrain of thrashers has been described (Fokidis and Deviche, 2012) and this information was used to locate the PVN and ME in this study. Images of the PVN and ME were digitized using a camera (Olympus DEI-750D) attached to a light microscope (Olympus BX60) at 40× magnification and using constant microscope, camera and computer settings. Images were analyzed blind with respect to feeding group. For each brain section, an 'out of focus' image was taken of an area devoid of immunolabeling to correct for background staining as described in Fokidis and Deviche (Fokidis and Deviche, 2012) using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Springs, MD, USA). Images were converted to black and white (Gray Scale 16 function) and flattened (Filter enhancement function). The 'out of focus' image was 'subtracted' from the image of interest (background correction function).

Four separate measurements were used to quantify differences in AVT-ir in the PVN between feeding groups: (1) the staining intensity of AVT-ir within a 100 µm diameter circular area of interest (AOI) centered over the PVN; (2) the cell density, or the number of AVT-ir cells within the 100 µm AOI; (3) the cell size, or the cross-sectional area of a subset (N=7 to 20 cells per section) of clearly delineated and non-overlapping AVT-ir cells; and (4) the cell staining, the optical density of the above individual cells. For each measurement, means from multiple sections including both left and right hemispheres from each bird were used in analyses. To quantify the staining within the ME (ME staining), the optical density of 10 circular AOIs (each 25 µm diameter) placed along the entire length of each representative image of the ME was determined and means were generated for each bird and used in further analyses. Further details on quantifying these variables can be found in Fokidis and Deviche (Fokidis and Deviche, 2012).

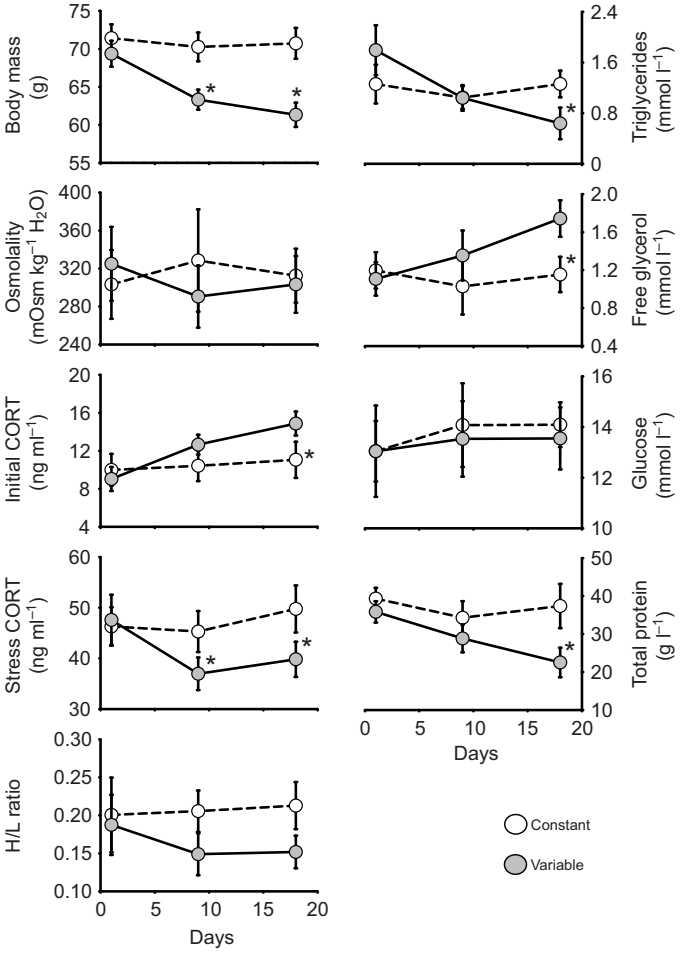

Fig. 1. Changes in body mass, osmolality, plasma corticosterone (CORT), blood heterophil to lymphocyte (H/L) ratio and plasma metabolites in curve-billed thrashers, *Toxostoma curvirostre* (N=27), in response to either a constant or variable feeding regime. Asterisks indicate a significant difference between variable and constant feeding groups (*P≤0.05).

Statistical analysis

Two-sample Student's t-tests were used to test whether the cumulative (summed over the duration of the study) food intake differed between variable and constant groups for both trials of the study. All data sets were tested for normality prior to analysis. H/L ratios and the proportion of time spent perching required arcsine square-root transformation to achieve normality prior to further analysis. Changes in body mass, plasma CORT, triglycerides, free glycerol, glucose, total protein, osmolality and the H/L ratio in response to feeding regime were assessed using repeated-measures analysis of covariance (RM-ANCOVA). Pearson's correlations between these variables revealed negligible multicolinearity (all P>0.13), thus supporting the use of an ANCOVA approach. The RM-ANCOVA models included feeding groups (variable *versus* constant) as the fixed factor, and study trial (1 *versus* 2) and bird origin (urban *versus* desert) as covariates along with all relevant interactions. *Post hoc* comparisons were performed using Tukey's honestly significant difference (HSD) tests. Comparisons of organ and muscle masses, AVT-ir (staining intensity, cell size, cell and ME staining), and behavioral data were carried out using ANOVA with feeding group, bird origin and trial number as between-subject factors. Body mass was entered into the model as a random factor, but was subsequently removed if not significant at P≤0.05. Tail feather length was compared between variable and constant groups using two-sample Student's t-tests. Fault bar numbers and AVT-ir cell densities in the two feeding groups were compared using non-parametric Mann–Whitney U-tests. All data are presented as means ± s.e.m. for parametric tests, and medians ± 95% confidence intervals (CI) for non-parametric tests. The critical alpha level for all tests was set at 0.05. Where multiple univariate comparisons were performed, a Bonferroni-corrected alpha value of ≤0.016 is also presented.

RESULTS

In both trials, birds in the variable and constant feeding groups consumed an equivalent amount of food (for trial 1, constant: 205.17±26.21 g, variable: 204.93±34.04 g, t_{25}=0.825, P=0.371; for trial 2, constant: 204.38±47.56 g, variable: 206.79±61.50 g, t_{25}=0.506, P=0.749; pooled: t_{52}=0.676, P=0.571). Thus birds ate more food when available, but the amount of leftover food was similar regardless of food available (Pearson's correlation between

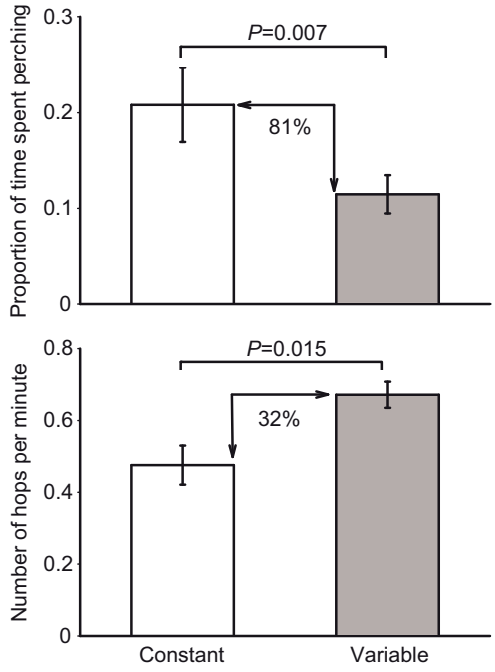

Fig. 2. Differences in two measures of behavioral activity between curve-billed thrashers, *Toxostoma curvirostre* (*N*=27), in response to either a constant or variable feeding regime.

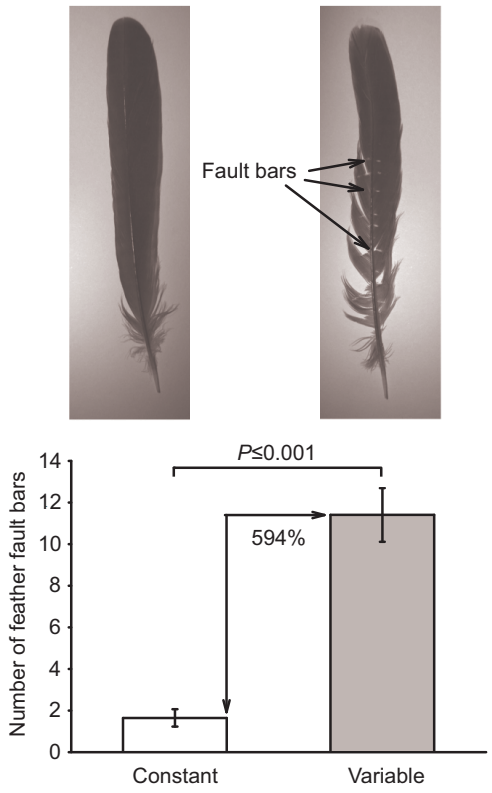

Fig. 3. Number of fault bars in re-grown tail feathers of curve-billed thrashers, *Toxostoma curvirostre* (*N*=27), in response to either a constant or variable feeding regime. Representative photos indicate variation in feather integrity between feeding groups.

food provided and consumed: r=0.637, P=0.015; food provided and leftovers: r=0.266, P=0.183). Neither origin of a bird (urban *versus* desert) nor trial order (first or second) significantly influenced any of the above variables during the study (all P>0.071). Thus data from both trials were pooled regardless of origin or trial order.

At the onset of the experiments, the variable and constant feeding groups did not differ with respect to body mass or any plasma variables (all P>0.092; Fig. 1). Birds in the variable feeding group decreased their body mass relative to birds in the constant feeding group ($F_{2,25}$=5.110, P=0.002; Fig. 1). Plasma osmolality did not change in response to feeding regime and was similar between the variable and constant feeding groups ($F_{2,25}$=0.483, P=0.395; Fig. 1). Birds in the variable feeding group elevated their initial plasma CORT, but only differed between the two groups only at the end (day 18) of the experiment ($F_{2,25}$=3.018, P=0.046; Fig. 1). In contrast, birds in the variable feeding group decreased their plasma CORT in response to 30 min of restraint stress compared with birds in the constant feeding group. This decrease was observed on days 9 and 18 ($F_{2,25}$=7.225, P≤0.001; Fig. 1). The variable feeding regime tended to decrease the H/L ratio although this was not statistically significant ($F_{2,25}$=1.920, P=0.067; Fig. 1).

Birds in the variable feeding group decreased their plasma levels of triglycerides ($F_{2,25}$=2.099, P=0.048; Fig. 1) and increased their free glycerol levels ($F_{2,25}$=2.444, P=0.037; Fig. 1) compared with those in the constant feeding group. However, in both cases *post hoc* tests revealed differences from controls only on the last day of the experiment (Fig. 1). By contrast, glycemia did not change during the course of the study or differ between birds in the variable and constant feeding groups ($F_{2,25}$=0.099, P=0.612; Fig. 1). However, total plasma protein concentration declined in the variable feeding group and differed significantly from that in the constant feeding group on day 18 ($F_{2,25}$=6.037, P=0.008; Fig. 1). All the above variables were similar between experimental trials (all P>0.103).

Thrashers in the variable feeding group spent 81% less time perched without engaging in activity than thrashers in the constant feeding group ($F_{1,26}$=5.141, P=0.007; Fig. 2). Birds in the variable feeding group were also more active, exhibiting 32% more hops than birds in the constant feeding group ($F_{1,26}$=3.005, P=0.026; Fig. 2).

All birds had completely re-grown their plucked tail feathers by the end of the study. The length of re-grown feathers did not differ between the variable and constant feeding groups (constant: 12.8±1.4 mm; variable: 13.1±2.6 mm; t_{25}=1.025, P=0.178). However, re-grown feathers in birds in the variable feeding group were in more ragged condition, especially along their proximal end, than those in the constant feeding group. This difference is reflected in a group difference in the number of fault bars (Mann–Whitney U=73.50, P=0.018; Fig. 3).

Birds in the variable feeding group also decreased the mass of their pectoralis muscles ($F_{2,21}$=4.179, P=0.002; Fig. 4), with no difference between left and right muscles (both P>0.306). However, variable feeding did not alter the mass of the liver ($F_{2,21}$=0.561, P=0.622), gizzard ($F_{2,21}$=0.937, P=0.307) or spleen ($F_{2,21}$=0.490, P=0.781). Variation in the above parameters was not associated with variation in body mass (all P>0.081).

Differences between birds in the variable and constant feeding groups were also observed in the expression of AVT-ir. Birds in the variable feeding group had 24% less staining intensity in the

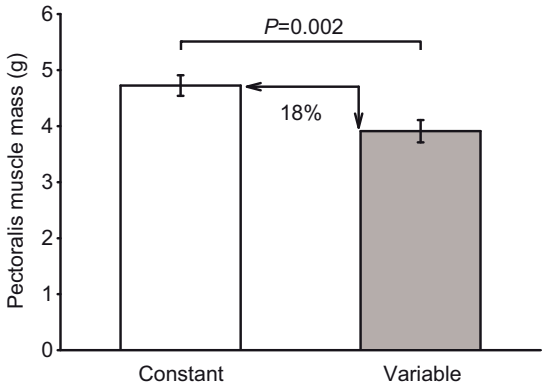

Fig. 4. Pectoralis muscle mass of curve-billed thrashers, *Toxostoma curvirostre*, fed a constant (N=13) or variable (N=14) amount of daily food.

PVN ($F_{2,21}$=7.115, P≤0.001), 16% smaller AVT-ir cells in the PVN ($F_{2,21}$=2.662, P=0.047) and 97% higher ME staining ($F_{2,21}$=8.931, P≤0.001) than birds in the constant feeding group (Fig. 5). No differences between groups was observed with respect to either the number of AVT-ir cells in the PVN (constant: 648.82±57.13 cells, variable: 671.11±50.04 cells; Mann–Whitney U=18.00, P=0.603) or the cell staining intensity (in arbitrary units, constant: 193.75±30.03, variable: 186.39±17.66; $F_{2,21}$=1.079, P=0.062).

DISCUSSION

Temporal variation in food availability is an often-cited factor influencing life-history traits (Reznick and Yang, 1993; Anderies et al., 2007; Mikolajewski et al., 2007), but few empirical studies have attempted to test the consequences of and adaptations to a temporally unpredictable food supply. We subjected captive curve-billed thrashers either to a constant (same amount of food each day) or to a variable (differing amounts of food each day) feeding regime and measured the behavioral and physiological effects of this manipulation to test two competing hypotheses. The adaptive regulation hypothesis proposes that during variable food availability, individuals decrease physiological functions that deplete energy reserves whilst conserving energy by minimizing behavioral activity. In contrast, a variable food supply may be viewed as an environmental stressor, thus promoting an enduring stress response. According to the chronic stress hypothesis, prolonged HPA activation results in depletion of energy reserves and promotes increased energetically costly behaviors that lead to mass loss and deteriorating health. In this study, thrashers belonging to the two experimental groups received and consumed equivalent amounts of food, thus differences between groups reflected temporal variability of food supplies and not total food intake. When exposed to a variable food supply for 18 days, thrashers lost mass, used more energy reserves (i.e. protein and lipids), increased their activity, altered their hypothalamic AVT-ir and increased their initial CORT levels. These birds also downregulated their plasma CORT response to handling stress compared with control birds. These observations are largely consistent with the predictions of the chronic stress hypothesis and provide limited support for the adaptive regulation hypothesis.

A variable food supply is physiologically stressful

Previous studies investigating responses to variable food supplies have used diverse experimental approaches and generated inconsistent results. Great tits, *Parus major*, subjected to variable durations of food deprivation increased their body mass more than birds that were food deprived for a constant duration, thus supporting the adaptive regulation hypothesis (Bednekoff and Krebs, 1995). A study manipulating the time available to feed revealed increased asymmetry in primary feather growth in European starlings, *Sturnus vulgaris* (Swaddle and Witter, 1994), but this manipulation did not affect plasma CORT or reproductive hormones in western scrub jays, *Aphelocoma californica* (Bridge et al., 2009). Interruptions of feeding have been shown to increase the body mass of great tits (Macleod and Gosler, 2006) and starlings (Bauer et al., 2011), but not zebra finches, *Taeniopygia guttata* (Dall and Witter, 1998). Based on these studies it is difficult to ascertain whether the perception of a food supply as being variable (i.e. unpredictable) results in physiological stress. The above studies also did not monitor actual food consumption and therefore did not separate the metabolic effects of actual food intake from the ‘perception’ component. In the present study, thrashers belonging to the two experimental groups consumed similar amounts of food during the experiment, indicating birds in the variable feeding group consumed more than their daily average when available (i.e. compensatory feeding).

Despite having a similar energy intake as the constant group, thrashers that received variable amounts of food showed an increase in initial CORT and a suppressed plasma CORT response to handling stress. An increase in initial CORT can reflect chronic stimulation of the HPA axis (Sapolsky et al., 2000; Romero and Romero, 2002). However, chronic stress may attenuate the HPA activity and thus decrease CORT secretion (Rich and Romero, 2005). Baseline (initial) and stress-associated CORT are involved in different physiological functions because of their interaction with different receptors (Sapolsky et al., 2000). The higher initial CORT levels in birds from the variable feeding group compared with birds in the constant feeding group may have enabled them to mobilize their internal energy reserves, which is consistent with the observed decline in body mass. The decrease in stress-associated CORT seen in thrashers in the variable feeding group may result from limits placed on adrenal production of CORT. Future studies utilizing pharmacological approaches could test for adrenal sensitivity to ACTH or the potential role of a negative feedback affect of CORT on the hypothalamus or pituitary gland in shaping stress physiology during unpredictable access to food.

To further investigate effects of unpredictable food availability on stress physiology, we considered an upstream component of the HPA axis, the production and secretion of AVT from the hypothalamus during the acute stress of capture and handling. Previous research identified the neural AVT system and its action on the pituitary gland as a source of variation in the HPA axis of free-living thrashers from urban and desert areas (Fokidis and Deviche, 2011; Fokidis and Deviche, 2012). Thrashers from these two habitat types show differences in plasma CORT, with urban birds having consistently a lower initial CORT level and a higher CORT response to handling stress than desert birds (Fokidis et al., 2009; Fokidis and Deviche, 2012), as well as increased ME staining intensity, i.e. increased release of AVT during capture (Fokidis and Deviche, 2012), than desert birds. The environment of urban Phoenix may act as a buffer to the seasonal variation in food availability characteristic of the desert, and thus a constant food supply was deemed to be compatible with an urban environment, and a variable food supply representative of a seasonal environment. The thrashers used in this study were captured from both urban and desert habitats, but time in captivity eliminates many of the differences (e.g. body mass, CORT secretion) present between the

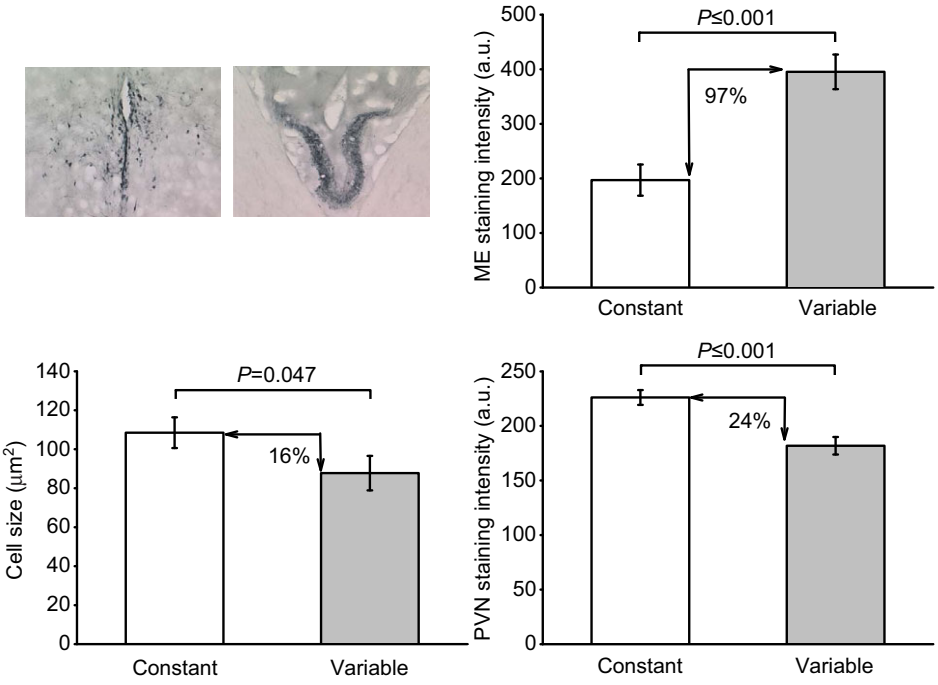

Fig. 5. Differences in immunoreactivity of arginine vasotocin in the paraventricular nucleus (PVN; left photo) and the median eminence (ME; right photo) of curve-billed thrashers, *Toxostoma curvirostre*, fed a constant (N=13) or variable (N=14) amount of daily food.

bird populations (Fokidis et al., 2011a). Similarly, bird origin did not influence any variable investigated in this study, suggesting that responses to treatment are plastic in nature (based on the current state) and not genetically predetermined. We manipulated food access in captive birds to determine whether thrashers viewed food variability as a chronic stressor, or are capable of physiological or behavioral strategies aimed at conserving energy. Thrashers subjected to variable food availability had higher ME staining intensity than birds with access to consistent amounts of food, and this observation is opposite to our predicted response, based on the wild bird research. However, replicating ecological conditions in captivity is inherently difficult, and the randomized availability of food in this study imperfectly mimics the seasonal variation in food resources in the desert, which are less stochastic in nature.

The most common interpretation of immunohistochemistry data is that immunostaining indicates a matching of peptide release with production (Panzica et al., 2001; Kabelik et al., 2008; Sewall et al., 2010) and thus greater ME staining would reflect a larger release of AVT into the hypophyseal portal system (Fokidis and Deviche, 2012). Based on this interpretation, the data are consistent with the higher initial CORT levels observed in the variable compared with the constant feeding group, and thus feeding treatment influenced the amount of AVT released during acute handling stress. Within the PVN, birds in the variable feeding group had smaller AVT-ir cells and lower staining intensity than birds in the constant feeding group. The former may be indicative of the recent release of AVT into the ME from the PVN during handling stress (Dawson and Goldsmith, 1997; Clerens et al., 2003), which is consistent with the greater ME staining. In this study we were unable to separate short-term changes in AVT immunostaining in response to handling stress from the longer-term effects of treatment.

Altogether, these observations are consistent with birds exposed to a variable food supply having higher initial CORT levels than birds with access to a constant amount of food. The secretion of CORT also impacts immune physiology including the H/L ratio. Chronic stress is considered to increase this ratio (Gross and Siegel, 1983; Vleck et al., 2000), but in free-living thrashers initial CORT is consistently negatively associated with this ratio (Fokidis et al., 2008). Nonetheless, feeding regime did not alter the H/L ratio in captive thrashers despite changes in CORT secretion, suggesting that H/L ratios may be influenced by other unknown factors worthy of study.

Energy expenditure drives changes in energy reserves

Thrashers exposed to a variable feeding regime decreased their body mass, total plasma proteins, triglycerides and mass of their pectoralis muscles. These observations suggest that variable access to food increased the usage of energy reserves. This is further supported by the increased concentrations of free glycerol in plasma, which is considered an indicator of triglyceride (i.e. lipid) breakdown (Guglielmo et al., 2002; Guglielmo et al., 2005), and the increased occurrence of fault bars during feather re-growth. The catabolic nature of CORT provides an explanation for these observations, as CORT encourages hepatic gluconeogenesis, which involves both lipid and protein breakdown to provide substrates for glucose production (Rahman and Clayton, 1981; Warne et al., 2009). Previous research has shown that feathers with fault bars contained more CORT than those without them (Bortolotti et al., 2009), but artificially increasing CORT levels through implants did not increase the presence of fault bars but altered other aspects of feather quality (DesRochers et al., 2009). Free glycerol increased with variable feeding, but glucose did not change during the course of the experiment. One interpretation of these findings is that glucose levels are tightly regulated (Braun and Sweazea, 2008) and thus gluconeogenesis was sufficient to keep pace with metabolic usage of glucose, resulting in stable (i.e. non-increasing) levels.

The decrease in body mass in thrashers exposed to a variable food source likely resulted mostly from the catabolism of muscle and not fat tissue because these birds presumably have limited capacity for fat storage (Bednekoff and Krebs, 1995; Witter and Swaddle, 1997; Pravosudov et al., 2001; Polo and Bautista, 2002). This conclusion is supported by two observations: (1) the decrease in mass of the pectoralis muscle, a predominant ‘power’ muscle involved in flight (Driedzic et al., 1993); and (2) no change in the

mass of the liver, used as a measure of hepatic lipid storage (Frayn et al., 2006), with feeding regime. During flight, avian muscles often rely on fatty acid catabolism for energy (Maillet and Weber, 2006), which can be further supplied by triglyceride breakdown. However, thrashers in the variable feeding group also exhibited a decline in total plasma protein compared with controls. Total plasma protein is thought to largely reflect plasma albumin and globulin levels (Cray et al., 2008; Roman et al., 2009), but high glycemia in birds may interfere with refractometry readings (Harr, 2002). As the feeding regime did not affect plasma glycemia, the decrease in the refractometer reading likely did not result from changes in plasma glucose. Previous research in starlings demonstrated that albumin can decrease in response to chronic stress (Awerman and Romero, 2010). Albumin contributes to maintaining the blood osmolality (Harr, 2002) but the decrease in plasma proteins in the variable feeding group was not associated with a change in plasma osmolality. Decreases in plasma albumin are typically due to metabolic clearance (i.e. loss to urine) or severe blood loss (Harr, 2002). These effects are unlikely to have occurred in the present study because birds excrete little protein (Harr, 2002) and thrashers remained in good health throughout the study.

Nutritional stress can deplete circulating globulins (Lynn et al., 2003; Lynn et al., 2010) and this may account for the decrease in total plasma protein observed in the variable feeding group. Understanding how avian blood proteins respond during stress and energy restriction is important to gain a clearer perspective on how energy reserves are utilized. The changes in plasma metabolites observed in this study suggest a direct effect of CORT on the usage of energy reserves, but the two experimental feeding regimes resulted in an equivalent energy intake. The equal energy intake was likely due to compensatory feeding by birds in the variable feeding group. Despite this apparent difference in daily food intake, no differences in gizzard mass were observed. Many species exhibit changes in the size of the gizzard in response to seasonal changes in food type or food quality (Walsberg and Thompson, 1990; Piersma et al., 2004) and intake (Van Gils et al., 2006). The lack of change in size of the gizzard may simply reflect the massive energetic cost associated with restructuring this large abdominal organ (Piersma et al., 2004; Van Gils et al., 2005) or perhaps the lack of sufficient food deprivation to induce such a change.

An alternative explanation for the increased usage of energy reserves in response to variable feeding is an increase in energy expenditure. Thrashers exposed to a variable food source increased their activity compared with controls. This increase may have resulted from elevated plasma CORT. Indeed, CORT in birds stimulates foraging behavior (Lynn et al., 2003; Lohmus et al., 2006; Angelier et al., 2007; Vaanholt et al., 2007; Bauer et al., 2011) and elevates perch hopping behavior in captive birds (Breuner et al., 1998). Indeed, in the present study, increased CORT may have mediated the compensatory feeding and increased activity (i.e. hopping) of thrashers in the variable feeding group. Many studies have examined how body mass responds to variability of a food source, but few studies have considered this behavioral component, which is also a strong determinant of energy balance, and hence body mass. Indeed, species that gain body mass with variable feeding may in part attain this by decreasing their activity and thus conserving energy.

Conclusions

We measured the hormonal, metabolic and behavioral responses of a non-migratory songbird to a variable (i.e. unpredictable) food source in the context of two competing hypotheses: (1) the adaptive regulation hypothesis, which predicts that birds exposed to variable feeding conditions will conserve current reserves by limiting energetically costly behaviors and physiological functions and attempt to build them up by increasing food intake; and (2) the chronic stress hypothesis, which predicts that food variability may induce a stress response, resulting in a mobilization of intrinsic energy reserves and thus a maladaptive decline in body mass. The present results show an increased usage of intrinsic energy reserves and changes in stress physiology in response to variable food supply and, therefore, largely support the latter hypothesis. However, instead of a direct effect of stress on energy reserves (as food consumption was controlled for), we observed an indirect effect mediated by increased energy expenditure. Many studies have considered the adaptive regulation to variable food abundance as a mechanism for persisting in habitats that are unpredictable by nature, such as those subjected to frequent inclement weather. Our research demonstrates that variables such as behavior, which are rarely considered in studies on this topic, may drive the observed patterns. It also provides evidence that differences in the ‘predictability’ of a food source between habitat types, across seasons, or even in response to landscape change, may be sufficient to have a negative physiological impact, even without altering the amount of food available. Studies investigating the role of food availability in establishing ecological patterns in free-living species will benefit from accounting for this temporal component.

LIST OF ABBREVIATIONS

ABC	avidin-biotin-chromagen
ACTH	adrenocorticotropin hormone
AOI	area of interest
AVP	arginine vasopressin
AVT	arginine vasotocin
AVT-ir	AVT-like immunoreactivity
CORT	corticosterone
CRH	corticotropin-releasing hormone
DFI	daily food intake
H/L	heterophil to lymphocyte ratio
HPA	hypothalamic–pituitary–adrenal
ME	median eminence
PB	phosphate buffer
PBT	phosphate buffer with triton-X
PVN	paraventricular nucleus

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