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Original investigation

Coping with environmental stress: The effects of wastewater pollutants on energy stores and leptin levels in insectivorous bats

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ABSTRACT

Anthropogenic environmental stress imposes increasing pressure on ecosystems. Wastewater treatment works (WWTWs) are major stressors in urban environments and are associated with high levels of pollutants that bioaccumulate and elicit stress responses in animals such as bats. Stress responses are linked with increased metabolic rate and may result in energy store depletion. In this study we measured the effects of WWTWs on energy stores and leptin levels in the insectivorous bat, *Neoromicia nana*. Energy stores including glucose, glycogen, lipids and proteins were measured in the storage tissues (liver and pectoral muscles) and blood of bats. Further, lactic acid was measured in the pectoral muscles to provide an indirect measure of anaerobic respiration and stress. Leptin, the hormone associated with satiation, and hypoxia inducible factor 1α (Hif-1α) were measured in the interscapular brown adipose tissue (iBrAT). Bats caught at WWTWs exhibited significantly higher pectoral glucose and lactic acid concentrations than bats at reference sites, indicating a stress induced increase in glucose demand and increased reliance on gluconeogenesis to fuel this response. However, glycogen, lipid and protein stores were not depleted in WWTW bats. This may be due to their fat-rich diet at WWTW sites. Moreover, this high polyunsaturated fatty acid (PUFA) diet may be responsible for the unexpectedly high leptin levels in WWTW bats. Lactic acid concentrations were elevated in the pectoral muscles of bats at WWTW sites, indicating some degree of oxidative stress although there was no increase in iBrAT Hif-1α levels. These results show that in the face of environmental stresses, *N. nana* is able to maintain energy stores.

Introduction

With rapid urbanisation and human expansion, animals are increasingly exposed to anthropogenic environmental stressors (Vitousek et al., 1997). One such stressor is wastewater treatment works (WWTWs), that introduce toxicants and pollutants such as metals, endocrine disrupting chemicals and microplastics into the environment through exposed settlement tanks and released effluent (Naidoo et al., 2013; Park et al., 2009). These pollutants can have severe negative impacts on the fitness of organisms (Bremner, 1998; Sánchez-Chardi et al., 2009). However, organisms that are able to exploit resources at WWTWs, yet tolerate exposure to associated toxicants, often thrive (Park and Cristinacce, 2006; Park et al., 2009). For example, pollutant-tolerant chironomid midges (Diptera) are highly abundant at WWTW sites, which, in turn, attracts insectivorous animals such as bats and birds (Naidoo et al., 2011, 2013; Park and Cristinacce, 2006). Although midges can survive in polluted environments, they accumulate toxicants in their bodies (Hare et al., 1991; Krantzberg and Stokes, 1990) that may have lethal or sub-lethal effects on their predators.

Sub-lethal effects of WWTW pollutant exposure can include a more pronounced immune response (Lum et al., 2007), changes in metabolic rate, and an inability to maintain energy stores of glycogen and lipids in exposed organisms (Martin et al., 2003; Zhang et al., 2012). Glucose, the major fuel for metabolic activity, is produced via glycogenolysis (the break-down of glycogen) and gluconeogenesis (the synthesis from pyruvate, glycerol, lactate and alanine) in the liver (Bollen et al., 1998; Srivastava and Krishna, 2010; Wallace and Barritt, 2002). With exposure to stress, these processes are upregulated to accommodate for the increased glucose demands of affected organs (Martin et al., 2003). However, as glycogen stores become depleted, lipid catabolism (lipolysis) is increased to breakdown fats into glycerol and free-fatty acids (Wallace and Barritt, 2002). As fat stores become depleted, muscle protein is catabolised to produce amino acids such as alanine (Wallace and Barritt, 2002). Gluconeogenesis can be upregulated due to stress, and is fuelled by metabolic end products such as

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alanine, glycerol and lactic acid (Bollen et al., 1998; Wallace and Barritt, 2002). Lactic acid, produced in muscle tissue via anaerobic respiration, may also be converted to glucose via gluconeogenesis in response to stressors (Himwich et al., 1930). Furthermore, the depletion of fat reserves when energy stores are compromised may lead to the down-regulation of the satiation hormone, leptin (Ahima and Flier, 2000; Geiser, 2004; Morris et al., 1994). One of the main functions of leptin in hibernating bats is to regulate fat storage and metabolic rate during the prehibernatory phase (Kronfeld-Schor et al., 2000). Because leptin is responsible for maintaining energy homeostasis, particularly in small mammals such as bats, the down-regulation of this hormone may result in a reduction in metabolism and the onset of torpor to conserve energy until stores can be replenished (Ahima and Flier, 2000; Geiser et al., 1998; Westman and Geiser, 2004; Morris et al., 1994).

Eliciting stress responses, such as a response to the pollutants associated with WWTWs, is energetically costly (Hochachka and Somero, 2002), and may affect key physiological processes of metabolic rate and energy utilisation. Chronic exposure to stressors, such as organochlorine exposure, can significantly increase the food intake and metabolic rate of the insectivorous bat *Pipistrellus pipistrellus* (Swanepoel et al., 1999). However, it is unknown whether bats may be able to cope with the long term exposure to pollutants whilst exhibiting higher metabolic rates.

The small insectivorous bat *Neoromicia nana* (Vespertilionidae, Chiroptera) often hunts midges at WWTWs, and consequently is exposed to metals such as cadmium, chromium and nickel (Naidoo et al., 2011, 2013, 2015). We have previously shown that foraging at WWTWs is detrimental to *N. nana.* More specifically, they exhibited increased haematocrit and DNA damage as well lesions in their livers and kidneys (Naidoo et al., 2015, 2016). This study aimed to determine the effects of foraging at WWTW on the energy stores and leptin levels of *N. nana*. We predicted that bats exposed to WWTW toxicants would rely on anaerobic respiration and exhibit depleted muscle and liver energy stores. We also predicted lower leptin levels compared to bats foraging at reference sites to facilitate lower energy expenditure during rest as an energy saving mechanism. In adipose tissues, leptin is under direct control of hypoxia inducible factor (Hif), the main modulator of oxygen homeostasis, and we therefore expected Hif-1α levels to be low in WWTW bats at rest.

Material and methods

We collected *N. nana* from four sites during April 2015: Verulam (29.646241°S 31.063543°E) and Umbilo (29.845283°S 30.890776°E) WWTWs; and Bufflesdrift (29.756730°S 30.678980°E) and Inkunzi Lodge (28.565201°S 31.241312°E) reference sites. Bats were captured with mist nets at WWTWs and by hand from roosts at reference sites. We identified bats to species using a taxonomic key (Monadjem et al., 2010), and released non-target animals at the capture site. Captured *N. nana* bats were sexed and life-stage was determined (adult or subadult), by examining the ossification of the finger bones (Kunz and Anthony, 1982). When possible, we collected five females and five males. We measured forearm length (to the nearest 0.1 mm) with vernier calipers [Ajax Scientific, Scarborough, Canada] and mass (to the nearest 0.5 g) with a Pesola scale (Lightline 10050, [Pesola, Schindellegy, Switzerland]). Bats were kept individually in cotton bags overnight to enter torpor, and were humanely euthanised (as approved by the University of KwaZulu-Natal Animal Ethics Committee, Reference: 014/15/Animal) the following morning. Dissected tissues were weighed and frozen in dry ice or liquid nitrogen before storage at −80 °C until analysis. Tissues associated with energy storage (liver and pectoral muscles) (Markan et al., 2010) were weighed and whole blood was collected. Interscapular brown adipose tissue (iBrAT) was collected to determine the expression of Hif-1α protein and the hormone leptin because this tissue is responsible for thermoregulation (Klaus, 2001). Body condition index (BCI) was calculated as body mass/forearm length (Speakman and Racey, 1986).

Tissue glucose, L-lactate, glycogen, lipid and protein concentration

Circulating blood glucose concentration in approximately 1 μl of blood was measured using a clinical glucometer (Diamond GD50 [FORA®, Moorpark, United States], > 95% accuracy, precission <5% [ISO 15197:2003]). To quantify liver and pectoral muscle D-glucose and L-lactate levels, 5 vols of 0.6 mol L^{-1} perchloric acid (PCA) was added to pectoral muscle and liver tissue respectively, homogenized (TissueLyser LT [Qiagen, Hilden, Germany]) and centrifuged (10 000 x *g*; 10 min; 4 °C). The supernatant was then used to measure pectoral muscle and liver glucose concentrations using a GOPOD D-Glucose assay (K-GLUC, Megazyme, Bray, Ireland), and pectoral muscle lactic acid concentration using an L-Lactate assay (K-LATE, Megazyme, Bray, Ireland), both according to the manufacturer's guidelines (K-GLUC and K-LATE manuals).

To enable the quantification of glycogen in glycosyl units, a subsample of the glucose homogenate was supplemented with 200 μl of 10 kU L^{-1} amyloglucosidase (Sigma, St. Louis, United States) and 10 μl of mol L^{-1} $KHCO_3$ (43 mmol L^{-1} in final reaction). After shaking for two hours at 40 °C, PCA (210 mmol L^{-1} in the final reaction) was added. The supernatant was then used to determine glycogen derived glucosyl content using a GOPOD D-Glucose assay (Megazyme, Bray, Ireland). The difference between initial glucose content and this value was then calculated to provide a measure of glycogen derived glucose (Vosloo et al., 2002).

Lipids were extracted from pectoral muscle and liver tissue (Bligh and Dyer, 1959). Samples were homogenized in a solution of PO_4 buffer, 96% methanol and 99.8% chloroform (4:10:5). This was then left on a shaker for 18 h. Thereafter, 10 ml of ultra-pure water and 99.8% chloroform solution (1:1) was added and the vials were vortexed. The samples were then left for 24 h to separate. The bottom layer was then removed and the chloroform was allowed to evaporate at room temperature in a fume hood, leaving only the dry lipids. Dry lipids were weighed, and calculated as a mass fraction of tissue mass. The protein concentration of the pectoral muscles and liver tissue was determined using the method described by Freitas et al. (2005): sub-samples of pectoral muscle and liver were homogenized in a 0.9% NaCl solution (TissueLyser LT [Qiagen, Hilden, Germany]). The samples were then centrifuged at 13 400 x *g* for 30 min at 4 °C and the protein concentration of the supernatant was quantified using a BCA Protein Assay (Pierce®, Los Angeles, United States).

Leptin and Hif-1α

To quantify leptin levels, hormone was extracted from iBrAT using a methanol solution (ultra-pure water and 96% methanol in a ratio of 1:2 respectively). A mouse leptin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Sigma-Aldrich, St. Louis, Missouri, United States) was used to detect and quantify the leptin hormone levels present in the iBrAT. Leptin amino acid sequences are highly conserved between taxa (Prokop et al., 2012). Further, slopes of the mouse leptin standard series and a dilution series of a *N. nana* leptin sample were not significantly different ($F_{1,7} = 0.169$; $P = 0.693$). Samples were diluted 40× (determined by a primary test run) and run in duplicate according to the manufacturer's instructions. Leptin concentrations were expressed relative to iBrAT mass. The extraction efficiency for the bat sample spiked with mouse lep-