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Haematological and genotoxic responses in an urban adapter, the banana bat, foraging at wastewater treatment works

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ABSTRACT

Wastewater Treatment Works (WWTWs) are a ubiquitous feature of the urban landscape. The Banana Bat, *Neoromicia nana* specifically exploits the high abundance of chironomid midge prey available at WWTWs but these populations also have higher levels of non-essential metals (Cd, Cr and Ni) in their tissues than bats foraging at unpolluted sites. Pollutant exposure may elicit primary physiological responses such as DNA damage and haematological changes. We investigated whether pollutant exposure from foraging at WWTWs impacts haematological and genotoxic parameters in *N. nana*. We compared four measures of haematological/genotoxic damage between *N. nana* foraging at three WWTWs and two unpolluted sites located in KwaZulu-Natal, South Africa: DNA damage measured by the Comet assay, total antioxidant capacity as indicated by the FRAP assay, chromosomal aberration indicated by micronuclei formation and blood oxygen capacity based on haematocrits. There was significantly higher DNA damage in *N. nana* at WWTWs than in bats from unpolluted sites, suggesting inadequate repair to double stranded DNA breaks. In addition, WWTW bats had a significantly lower antioxidant capacity than bats from unpolluted sites. This suggests that bats at WWTWs may have a diminished capacity to cope with the excess reactive oxidative species (ROS) produced from pollutants such as metals. There was no increase in micronucleus frequency in WWTW bats, indicating that cellular functioning has not yet been disrupted by chemical exposure. Haematocrits, however, were significantly higher in WWTW bats, possibly due to erythrocyte production in response to certain pollutants. Thus, effects of pollutant exposure in bats foraging at WWTWs elicit sub-lethal haematological and genotoxic responses which may pose serious long-term risks. This provides evidence that WWTWs, that are aimed to remove pollutants from the environment, can themselves act as a source of contamination and pose a threat to animals exploiting these habitats.

1. Introduction

To cater for the rapidly growing human population, natural land is being transformed into urban habitat at an alarming rate (McKinney, 2006). By 2050, nearly 70 percent of the global human population will be residing in urbanized habitat (United Nations, 2011). As a result, wildlife is becoming increasingly exposed to the physical features associated with urban development, and is showing a general decline in response to it (Vorosmarty et al., 2010). Wastewater Treatment Works (WWTWs) are a ubiquitous and often permanent component of the urban landscape. They receive both industrial and household waste which contains a cocktail of pathogens, inorganic and organic contaminants (Gagnon and Saulnier, 2003). The influent undergoes various stages of treatment in large, open-top sludge tanks before finally being discharged into rivers. During this process, the wastewater in sludge tanks is exposed and freely accessible to volant animals. In addition, pollution-tolerant chironomid midges thrive at WWTWs (Boonstra et al., 2009). Because chironomid midges at WWTW tanks are in direct contact with the wastewater, volant, insect-eating predators such as bats are at a high risk of contaminant intake and accumulation.

The Banana Bat, *Neoromicia nana* (family Vespertilionidae), is an urban adapter (Jung and Kalko, 2011) that exploits the high abundance of chironomid midge prey available at WWTWs (Naidoo et al., 2013). *N. nana* have a significantly higher abundance and feeding activity at WWTWs than unpolluted sites within the urban landscape. In addition, chironomid midges are the dominant prey type in the diet of these resident WWTW bats (Naidoo et al., 2013). Midges are however pollution-tolerant (Vermeulen, 1995). More specifically, midges can accumulate high levels of metals, without decreases in survival and growth to the adult stage (Al-Shami et al., 2010). Further, bioaccumulation of metals is characteristically more prominent in carnivorous small mammals than in herbivorous small mammals (Alleva et al., 2006; Hamers et al., 2006). Hence, *N. nana* foraging at WWTWs contain higher levels of non-essential metals (Cd, Cr and Ni) in the tissues than bats foraging at unpolluted sites

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(Naidoo et al., 2013). Of the tissue types analysed, metal levels in the kidneys correlated to metal levels at the polluted sites. The kidney is actively involved in metal regulation and detoxication, thus concentrations of toxic metals in the tissue reflect exposure and accumulation of those metals for a prolonged period (McGeer et al., 2000).

When organisms are exposed to pollutants, a cascade of response events is induced. DNA damage and haematological changes may occur primarily, followed by longer-term damage such as lesions in detoxication organs and ultimately, visible pathological disease. Various pollutants, including metals found in wastewater, are genotoxic agents which cause direct or indirect damage to genetic material. Direct genotoxic effects include chromosomal aberrations such as micronucleus formation and DNA damage such as strand breaks, adduct formation, protein cross-links and oxidative damage from reactive oxygen species (ROS) produced during metal interactions (Shugart, 2000).

Sub-lethal physiological responses such as chromosomal aberrations and DNA damage from contaminants are typically investigated in the laboratory in dose–response exposure experiments (Swanepoel et al., 1999). Few studies have investigated mammal responses to pollutant levels occurring in the environment, many of which focus on acute poisoning events (Kohler and Triebskorn, 2013) and mine pollution (Johnson et al., 1978; Sanchez-Chardi et al., 2008). For instance, physiological effects, including changes to haematological parameters, histopathological alterations, genotoxicity, and compromised enzymatic activity were noted in shrews (*Crocidura russula*) inhabiting an abandoned pyrite mining site (Sanchez-Chardi et al., 2008). Similarly, Zocche et al. (2010) found that bats (*Tadarida brasiliensis*) inhabiting coal-mines in Brazil had significant DNA damage.

Acute poisoning events including pesticide applications have resulted in several cases of increased mortality in bat populations (Clark et al., 1978; Kunz et al., 1977). Furthermore, high concentrations of organic pollutants were found in bats affected by White-nose syndrome, an emerging disease which is decimating North American bat populations (Kannan et al., 2010). Pollutant exposure in bats may thus contribute to immunosuppression (Pilosof et al. 2013), further increasing their susceptibility to infection by White-nose syndrome and other diseases. Physiological effects on bats foraging at WWTWs over a long period of time have not, however, been investigated. Understanding these effects is of critical importance because WWTWs are relatively common fixtures scattered across urban landscapes, and are intensively utilized by these animals.

The aim of this study was to therefore investigate whether pollutant exposure from foraging at WWTWs impacts haematological and genotoxic parameters in *N. nana*. We selected three measures of haematological/genotoxic damage which are relatively quick and cheap to perform and commonly used in routine assessments of ecotoxicological responses to environmental pollution: DNA damage measured by the Comet assay, chromosomal aberration indicated by micronuclei formation and blood oxygen capacity based on haematocrits. In addition, we measured muscle antioxidant capacity using the FRAP assay as a first-tier indication of tissue reducing power. We predicted that *N. nana* foraging at WWTWs would have a greater extent of DNA damage, compromised total antioxidant capacity, higher levels of chromosomal aberration and changes in blood oxygen capacity compared to bats foraging at unpolluted sites.

2. Methods

2.1. Sample collection

We collected pollutant exposed *N. nana* samples at three WWTWs which use sludge tank systems and contain high concentrations of wastewater-associated metals (lead, cadmium, chromium, nickel, copper, zinc and iron; Naidoo et al., 2013) in Durban, South Africa (S29°58′; E30°57′): Umbilo Wastewater Works (S29°50.44′; E30°53.31′), the Verulam Wastewater Works (S29°38.38′; E31°03.49′), and the Kingsburgh Wastewater Works (S30°04.29′; E30°51.26′) (Fig. 1). We selected two unpolluted reference sites in the Umdoni Park, Pennington about 80 km south of Durban (Fig. 1). Umdoni Park covers an area of 210 ha comprising mainly dense coastal forest representative of the Indian Ocean Coastal Belt biome (Mucina et al., 2006). There are no WWTWs located in the immediate vicinity of the park, with the closest WWTW situated > 8 km away. We sampled two sites within the forest: Unpolluted site 1 (S30°40.36′; E30°23.31′), located close to the border of the park, and unpolluted site 2 (S30°41.15′; E30°23.35′) located further inside the park. Because *N. nana* has a relatively small home range – 300 m from the roost (LaVal and LaVal, 1977) – individual turnover between unpolluted sites and contamination from the nearest WWTW was unlikely.

N. nana were captured with mist nets at WWTW sludge tanks, and both mist nets and harp traps at the unpolluted sites. All bats were collected during the summer (January–March 2013). Captured bats were sexed and their life-stage (juvenile or adult) was determined from the presence of cartilaginous epiphyseal plates (Anthony, 1988). Only adult bats were kept for analyses. We measured forearm length (to nearest 0.1 mm) with digital callipers, and body mass with a Pesola scale (to nearest 0.5 g). Species were identified using a taxonomic key (Monadjem et al., 2010) and species other than *N. nana* were released where they were caught. Captured *N. nana* were humanely euthanized, as approved by the University of KwaZulu-Natal Animal Ethics Committee (Reference: 031/13/Animal). Twenty μL of whole peripheral blood from each bat was immediately diluted with ethylenediaminetetraacetic acid (EDTA) (1:1) and stored on ice to prevent coagulation.

2.2. DNA damage

We assessed DNA damage using the single cell gel electrophoresis assay, or Comet assay, as described by Tice et al. (2000). The comet assay is a reliable method employed in genetic toxicology, which allows the quantification of DNA strand breakage or potentially pre-mutagenic lesions from exposure to toxic chemicals (Fontanetti et al., 2010). The basic steps of the comet assay are slide preparation, lysis, electrophoresis, neutralization and staining (Fairburn et al., 1995; Tice et al., 2000).

Frosted glass microscope slides (two slides per individual), modified to create two clear windows, were coated with 300 μL of 1 percent high melting point agarose (HMPA) and allowed to dry. Twenty μL of the blood/EDTA solution was added to 300 μL of 0.5 percent low melting point agarose (LMPA) kept at 42 °C. The HMPA-coated slides were then covered with 130 μL of the cell LMPA suspension and placed on ice to set. Prepared slides were placed in lysis buffer (2.5 mol L^{-1} NaCl, 0.1 mol L^{-1} EDTA, 1 percent Triton X-100, 1 percent DMSO) for three weeks at 4 °C. Subsequent to the lysing period, slides were rinsed with distilled water for 3–5 min. To allow alkali unwinding of the DNA, the slides were incubated in electrophoresis buffer (0.3 mol L^{-1} NaOH, 1 mmol L^{-1} EDTA) for 20 min in a horizontal gel electrophoresis tank. The unwound DNA was electrophoresed at a voltage of 25 V and a current of 300 mA for 20 min. Slides were rinsed with distilled water for 3–5 min and soaked in a cold, freshly prepared neutralization buffer (0.8 mol L^{-1} Tris–HCl buffer—pH 7.5) for 15 min.

Slides were then removed from the neutralization buffer, rinsed again with distilled water for 3–5 min and stained in 0.01 mmol L^{-1} ethidium bromide. After a final rinse in distilled water (3–5 min), slides were stored at 4 °C in dark, moist conditions. Slides were rehydrated with distilled water prior to imaging. Images of 100 cells per individual were captured with a Nikon E5400 camera, using a fluorescent microscope (Nikon Eclipse E400 microscope; magnification = 400 ×, filter B-2 A: excitation = 450–490 nM, barrier = 520 nM).

We used CASP 1.2.3b (CASPLab.com, 2010) software for image analysis. Nuclear material is observed as a comet, with the high-molecular-weight DNA contained in the head of the comet and the comet tail containing broken fragments (Olive and Banath, 2006) (Fig. 2a). We measured percent tail DNA and the olive tail moment (OTM) per cell (Fig. 2b). The OTM is a robust indicator of damage, providing a measure of the combination of head DNA, tail DNA and distribution of DNA in the tail (Kumaravel and Jha, 2006). We classified each cell into one of five damage categories based on percent tail DNA, according to Gorbi et al. (2008): class 1: < 5 percent; class 2: 5–20 percent; class 3: 20–40 percent; class 4: 40–95 percent; class 5: 95–100 percent.

2.3. Total antioxidant capacity (TAC)

We measured the total antioxidant capacity in *N. nana* pectoral muscle using the FRAP assay (ferric reducing/antioxidant potential) following Griffin and Bhagooli (2004) with modifications. The FRAP assay uses the reducing potential of antioxidants to produce a colour change from a reaction with ferric tripyridyltriazine (Fe^{III}-TPTZ), resulting in ferrous tripyridyltriazine (Fe^{II}-TPTZ) (Griffin and Bhagooli, 2004). Frozen pectoral muscle tissue was dissected and weighed before performing a whole cell extract using an extraction buffer as in Mosser et al. (1988). Tissue was disrupted in a TissueLyser (Qiagen) and then centrifuged for 10 min at 8050 × *g*. We quantified protein concentrations using a Thermo Scientific BCA