THE EFFECT OF WASTEWATER TREATMENT WORKS ON FORAGING ECOLOGY, HAEMATOLOGY, DETOXIFICATION ORGANS AND REPRODUCTION IN AN URBAN ADAPTER, THE BANANA BAT (*NEOROMICIA NANA*)

by

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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ABSTRACT

Natural land is rapidly becoming urbanized. Wastewater Treatment Works (WWTWs) are a ubiquitous component of this urban landscape. WWTWs may provide profitable foraging areas for insectivorous bats because of their association with a high abundance of pollution-tolerant chironomid midges (Diptera). However, bats that feed on these insects may also accumulate metal pollutants in their tissues, with acute or chronic effects on their health. There have been no studies to investigate whether African bats utilize these WWTWs as foraging grounds, and the potential physiological impacts from foraging at such sites. The aim of this study was to investigate the impact of WWTWs on foraging ecology and multiple tiers of physiology (haematology and genotoxicity, detoxification organs and reproduction) in an urban adapter, the banana bat (*Neoromicia nana,* family Vespertilionidae) in KwaZulu-Natal, South Africa. *N. nana* exhibited a significantly higher abundance and feeding activity at wastewater-polluted sites than at unpolluted reference sites. Additionally, the most abundant insect order at wastewater-polluted sites and in the diet of resident bats was Diptera, compared to a diverse insect diet at unpolluted sites. Thus, WWTWs provide an optimal food resource to bats in the short-term. However, I found significantly higher levels of essential and non-essential metals at WWTW-polluted sites, and in the tissues of WWTW bats than at unpolluted sites. Further, I found sub-lethal haematological and genotoxic responses related to increased metals in WWTW bats. Specifically, *N. nana* at WWTWs had significantly lower antioxidant capacity and significantly higher levels of DNA damage and haematocrits than bats from unpolluted sites. An accumulation of DNA damage, especially from double-stranded breaks ultimately leads to tissue damage and disease. These longer-term effects of chronic pollutant exposure should be most

evident in the organs involved in detoxification, the liver and kidneys. Indeed, I found evidence of disrupted balance of essential metals and mineral nutrients, histopathological tissue damage and whole organ effects in the liver and kidneys. Finally, I found reproductive system alterations in male *N. nana* at WWTWs. Although I did not find significant effects on the sex organs, testosterone hormone concentrations were significantly lower in male *N. nana* at WWTWs than in males from unpolluted sites. In addition, body condition indices for *N. nana* from the WWTWs were significantly lower than at unpolluted sites, suggesting lower quality male bats at WWTWs. Taken together, these results suggest the potential for serious long-term health risks, negative fitness implications and ultimately, population effects for these top predators within the urban landscape.

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville campus, from January 2009 to December 2011, under the supervision of Dr. M. Corrie Schoeman and co-supervision of Dr. Robin L. Mackey and Dr. Dalene Vosloo, and from January 2012 to December 2015, under the supervision of Dr. M. Corrie Schoeman and co-supervision of Dr. Dalene Vosloo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text. This thesis is presented as a compilation of published papers and unpublished manuscripts.

DECLARATION 1 - PLAGIARISM

I, Samantha Naidoo declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis:

Publication 1: Naidoo, S., Vosloo, D., Schoeman, M. C., 2013. Foraging at wastewater treatment works increases the potential for metal accumulation in an urban adapter, the banana bat (*Neoromicia nana*). African Zoology 48(1), 39 - 55.

Author contributions: SN led the writing, and collected and analysed the data; SN and MCS conceived the ideas and experimental design; MCS and DV contributed to the writing. The paper is published.

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TABLE OF CONTENTS

Page

Title page i

Abstract ii

Preface iv

Declaration 1 – Plagiarism v

Declaration 2 – Publications vi

Chapter 1 Introduction 1

1.1. Urban Pollution 2

1.2. Pollution through the trophic levels 4

1.3. Pollution effects on physiology 5

1.3.1. Genotoxicity and haematological responses 5

1.3.2. Effects on detoxification organs 6

1.3.3. Endocrine disruptors and reproduction 6

1.4. Bats as a model taxon 7

1.5. *Neoromicia nana* – the urban adapter 9

1.6. Outline of thesis 10

1.7. References 11

Chapter 2 Foraging at wastewater treatment works increases the potential for metal accumulation in an urban adapter, the banana bat (*Neoromicia nana*) 19

Abstract 20

Introduction 20

Methods 21

Results 25

Discussion 30

References 34

Chapter 3 Haematological and genotoxic responses in an urban adapter, the banana bat, foraging at wastewater treatment works 37

Abstract 38

Introduction 38

Methods 39

Results 41

Discussion 42

References 43

Conference contribution: Poster presentation 46

Chapter 4 Pollutant exposure at wastewater treatment works affects the detoxification organs of an urban adapter, the banana bat 48

Abstract 49

Introduction 49

Methods 50

Results 53

Discussion 53

References 56

Appendix A. Supplementary data 59

Chapter 5 Sex and the city: Reproductive system alterations in an urban adapter, the banana bat, exposed to endocrine-disrupting chemicals at wastewater treatment works 62

5.1. Abstract 63

5.2. Introduction 63

5.3. Methods 66

5.3.1. Sample Collection 66

5.3.2. Testosterone Concentration 67

5.3.3. Baculum Morphometrics 67

5.3.4. Gonadosomatic Indices (GSI) 68

5.3.5. Body Condition (BCI) 69

5.3.6. Statistical Analyses 69

5.4. Results 70

5.4.1. Testosterone Concentration 70

5.4.2. Baculum Morphometrics 70

5.4.3. Gonadosomatic Indices (GSI) 71

5.4.4. Body Condition (BCI) 72

5.5. Discussion 73

5.6. References 78

Chapter 6 Synthesis and conclusions 86

6.1. Synthesis/ Conclusions 87

6.2. Potential consequences for *N. nana* populations 90

6.3. Potential consequences for the local ecosystem 91

6.4. Future work 92

6.5. References 94

Acknowledgments 98

Chapter 1

Introduction

CHAPTER 1: Introduction

1.1. Urban Pollution

By the year 2050, the global human population is projected to pass 9 billion (United Nations Population Fund, State of World Population, 2014). Concomitantly, the expansion of cities is increasing rapidly, with large areas of natural land being transformed into urbanized landscapes (McKinney, 2006; Seto et al., 2011). It is expected that the majority of this urbanization will occur in coastal countries such as South Africa, where anthropogenic stress on the environment is already widespread (McKinney, 2006). To cater for the growing human populations, industrial development and services are increased. However, this development creates serious habitat alteration, which impacts on both the environment and its resident organisms. 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). The physical manipulation of the landscape by anthropogenic activities such as fragmentation has had predominantly negative impacts on the resident fauna and flora (Schmiegelow and Monkkonen, 2002).

In addition to the physical land-transformation, a chief anthropogenic disturbance to the urban environment is pollution. Pollution often has adverse effects on biodiversity (Azrina et al., 2006; Nedeau et al., 2003; Vörösmarty et al., 2010). Pollutant exposure may directly affect organisms or may influence them through modifications to the habitat or prey. River pollution is currently a major problem, and there has been a recent influx of data highlighting the poor state of South African rivers in urban areas (eThekwini Municipality State of the Rivers Report, 2011). Within the previous century, the species diversity of aquatic invertebrates (Williams et al., 2003) and aquatic vertebrates (Reash and Berra, 1987) has decreased significantly in polluted rivers. For instance, the global diversity of non-marine mollusks has been steadily decreasing, with 708 freshwater mollusc species included in the 2002 IUCN Red List of Threatened Species (Lydeard et al., 2004). Vertebrates exposed to river contaminants, such as the African bullfrog (*Pyxicephalus adspersus*), have shown a rapid decline in numbers in urban reserves in South Africa (Oberholster et al., 2008). The rapid rate of urbanization in South Africa has resulted in the introduction of a range of contaminants into freshwater ecosystems (Gleick, 1998). Increased industrial development produces inorganic and organic pollutants, such as chemical runoff from factories and sewage effluent that are deposited into rivers (Sacks and Buckley, 1998). Furthermore, industrial and pharmaceutical

substances containing xenobiotic chemicals such as endocrine disrupting chemicals (EDCs) are found in specific pollution sources such as wastewater (Fossi et al., 2002).

Wastewater treatment works (WWTWs) are ubiquitous in urban landscapes and are constantly in operation. Wastewater treatment works are an essential service, linked to urbanization and the concentration of human populations associated with it. They also have a precise point of effluent discharge into the river, allowing for a clear partitioning of sites receiving or not receiving wastewater. Furthermore, the type of pollution can be assessed from identifying wastewater constituents (Leland et al., 1974). Thus, WWTWs provide an ideal model to investigate the toxic effect of river pollution on exposed organisms.

Wastewater effluent contains both industrial and domestic input including solids, pathogens and organic and inorganic pollutants (Gagnon and Saulnier, 2003). Various types of operational practices are employed by wastewater treatment plants to treat waste effluent. Conventional operational practices such as the screening and spraying of wastewater onto percolating filter beds have been largely replaced by more recently designed systems (Govender, 2002). The system most often used in South Africa is the process of activated sludge, because it is able to cater for larger populations and requires small land space (Govender, 2002). Domestic and industrial waste influent received by treatment plants employing this system, undergoes an aerobic biological process whereby wastewater is degraded using microbial communities (Lalbahadur, 2005). The wastewater is treated in aeration and settling tanks. It is then chlorinated to remove pathogenic organisms before being discharged into rivers (Jackson et al., 2002). However, the sludge produced in the tanks used for this process contains high levels of metals (Braum, 2004; Govender, 2002). These metals, including lead (Pb), cadmium (Cd), chromium (Cr) and nickel (Ni), are particularly toxic to living organisms when ingested in large quantities or over a long period of time (Andres et al., 2000).

The processes used to treat wastewater focus on the removal of solids, nutrients and pathogenic bacteria, often neglecting the treatment of metals (Gagnon and Saulnier, 2003). In addition to the inefficient removal of metals from the wastewater, are varying efficiencies of EDC removal from wastewater according to the hydrophobicity and molecule size of the specific chemical (Nakada et al., 2006). Thus, effluent contains high levels of certain chemicals even after treatment (Janex-Habibi et al., 2009). Furthermore, organic waste that contributes a high input from nutrients such as nitrogen (N) and phosphorous (P) compounds, promote eutrophication where elevated quantities of organic matter are produced (Yount and Crossman, 1970). Because of changes to dissolved oxygen levels, this eutrophication commonly leads to altered invertebrate communities (McGarrigle, 1998).

1.2. Pollution through the trophic levels

Macroinvertebrate community structure at water bodies is highly related to water quality. As pollution levels increase, invertebrate diversity usually decreases (Mason, 2002), and the community becomes dominated by species with lower dissolved oxygen demands (McGarrigle, 1998). With lower levels of competition from more sensitive species, these pollution-tolerant insects are able to flourish at polluted sites. For instance, pollution-tolerant insect groups from the Chironomidae, Oligochaeta, Erpobdellidae, Ancylidae and Lymnaeidae were associated with sites located downstream of sewage outputs along Irish rivers (Abbott et al., 2009). Similarly, the two pollution-tolerant insect groups most associated with WWTWs in South Africa were Chironomidae and Oligochaeta (Dickens and Graham, 1998). In contrast, pollution-sensitive taxa (Ephemeroptera, Plecoptera, Trichoptera) were more closely associated with upstream sites along the same rivers (Abbott et al., 2009).

The shift in insect diversity at polluted sites compared to unpolluted sites in turn affects the prey available to predators within these habitats. For instance, in Italy, the European dipper, (*Cinclus cinclus*) is a semi-aquatic insect-eating bird that specifically preys on pollution-sensitive taxa (Sorace et al., 2002). As river quality has been decreasing, dipper populations have shown a corresponding decline (Sorace et al., 2002). In contrast, three-spined stickleback (*Gasterosteus aculeatus*) exploit the abundance of pollution-tolerant benthic oligochaetes in contaminated freshwater systems (Egeler et al., 2001). Thus, the abundance of some predators are negatively affected, whereas other predators may exploit the availability of prey. Although some may benefit, calculated bioconcentration ratios indicate that metal and some organic pollutants bioaccumulate in even higher concentrations in the predators of the affected insects (Hsu et al., 2006). However, in terrestrial food chains, biomagnification of metals, for instance, varies according to pollutant assimilation and immobilization in the predator species (reviewed in Laskowski (1991)). In addition, the transfer of pollutants through the food chain may be greater in specific types of contaminated prey, particularly pollution-tolerant insect species that are also capable of accumulating metals without impact (Hare, 1992).

The group of aquatic flies known as the chironomid midges are a prominent insect group capable of enduring polluted environments (Postma et al. 1995) and are amongst few other insect groups able to accumulate metals without being affected (Groenendijk et al., 1998). The growth rate of

chironomids is rapid, with a quick generation turnover, ensuring constant availability to the ecosystem (Menzie, 1981). Although chironomid larvae are found in sediment, the chironomid life cycle is dominated by the adult stage which is spent on and above the water surface (Ristola, 2000). At WWTWs in particular, chironomid midges often occur at a high density at artificial tanks containing wastewater (Broza et al., 2003). In fact, chironomid swarms are a characteristic feature at WWTWs.

In the wastewater, particulate matter upon which the midges feed contain various metals (Stuijfzand et al., 2000). Chironomid midges show a remarkable metal-regulating capacity, and can take up metals, distributing them in various parts of their body without negative effects on survival (Boonstra et al., 2009; Krantzberg and Stokes, 1990). In addition, Park et al. (2009) found that amongst other aerial invertebrates developing on sewage filter beds, chironomid midges take up a range of organic pollutants such as 17a-ethinylestradiol and butylated hydroxy aniline. When predators such as bats or birds constantly exploit this contaminated food source, the pollutants accumulate within the body. Unable to undergo metabolism, most metallic elements that are not excreted, become stored in tissue (Fritsch et al., 2010). In addition, the persistent lipophilic nature of organic pollutants causes them to accumulate in fat stores (Fossi and Marsili, 2003). In turn, the physiology and anatomy of the predator may be negatively impacted, with acute or chronic effects (Walker, 1998).

1.3. Pollutant effects on physiology

Acute exposure to pollutants can be lethal or elicit visibly observable effects (Yen et al., 2002). Numerous laboratory experiments have tested this by determining the lethal dose (LC_{50}) of specific pollutants in different animal species (Jolly et al., 1978). However, longer-term chronic exposure to pollutants, like predators may experience by exploiting pollution-tolerant prey, often result in sub-lethal effects in different levels of biological organization.

1.3.1. Genotoxicity and haematological responses

The first level where pollutant-induced sub-lethal damage may be evident is in the blood cells, which have a fairly rapid turnover rate. Genotoxic agents such as metals may cause DNA strand breaks, chromosomal aberrations, and other oxidative damage from reactive oxygen species (ROS)

produced during metal interactions (Shugart, 2000). In addition, toxic metals commonly found in WWTWs, including cadmium, nickel and lead, inhibit DNA repair at low concentrations. Although antioxidant systems such as superoxide dismutase scavenge free radicals to counteract damage from ROS production, wastewater-associated metals have the ability to impair antioxidant activity itself (Beyersmann and Hartwig, 2008) or form highly reactive radicals when they interact with the intermediates of ROS detoxification (Pham et al., 2013; Simpson, et al., 1988). If there is not enough protection from antioxidants against ROS, then cellular functioning may also be disrupted by pollutant exposure. Pollutants may interfere with cell division resulting in lagging chromosomic fragments, forming micronuclei in mammalian erythrocytes (Hartmann et al., 2008). In addition, erythrocyte volume may be affected, causing altered haematocrits. For instance, metal exposure lowers the haematocrit in wood mice, *Apodemus sylvaticus* (Rogival et al., 2006).

1.3.2. Effects on detoxification organs

Sub-lethal damage from pollutant exposure can cause long-term effects in various organs. However, the effects of ongoing exposure to pollutants are often most evident in the organs responsible for detoxification, the liver and kidney (Clark and Shore, 2001). Pollutants ingested by an organism are metabolized, excreted, accumulated or stored in a less toxic form (Baker et al., 2003). There are various mechanisms by which this may occur. To detoxify metals for instance, metallothionein proteins produced in the liver and kidney bind to the metal ions, preventing excess damage (Sakulsak, 2012). Thus, animals exposed to excess metals often have upregulated metallothionein levels (Dai et al., 2013; Sakulsak, 2012). However, pollutants may still accumulate in organ tissue leading to histopathological lesions, which can in turn alter organ size and function (Ma, 1989).

1.3.3. Endocrine disruptors and reproduction

Together with the risk for detoxification organs posed by pollutants at WWTWs, are other threats to physiological function. WWTWs receive high quantities of various EDCs, which are removed from treated wastewater in varying efficiencies (Huang and Sedlak, 2001). The endocrine system maintains hormonal balance. However, the action of endogenous hormones such as estrogen and testosterone may be modulated, mimicked, enhanced, or inhibited by EDCs, which bind to hormone receptors (Tyler et al., 1998). EDCs found in wastewater can therefore severely alter the reproductive physiology and behaviour of the resident wildlife exposed to them. Pollutant-induced

cases of intersex, altered primary and secondary sexual organs, and changes in sexual behaviour have been observed across taxa, including frogs, fish, birds and mammals (Tyler et al., 1998). This is because the development of sexual traits and sex organs like the testis and baculum, are mediated by hormones (Yonezawa et al., 2011). Moreover, disruptions to the sex organs and hormones affect an individual's ability to acquire mates and reproduce, and thus impact fitness. Ultimately, reproductive system alterations will carry longer term consequences for wild populations exposed to pollutants.

1.4. Bats as a model taxon

The effects of pollution on ecology and physiology have been studied in various animal groups. In mammals, a large majority of studies have been performed under laboratory conditions on non-volant-mammal species, particularly rodents. However, compared to similarly sized non-volant small mammals, bats have relatively long life spans (Barclay and Harder, 2003). The long life span of bats is a valuable characteristic in pollution studies, because the accumulation of certain metals such as cadmium, is specifically associated with age (Walker et al., 2002; Walker et al., 2007). Organic contaminants may also persist in bat tissue several years after exposure (Bayat et al., 2014). In addition, the slow reproductive rate of bats allows for clear trends of population decline or increase to be elucidated (Jones et al., 2009).

The life history characteristics of bats therefore render them excellent indicators of environmental quality (Jones et al., 2009). For instance, certain bats co-exist with humans in altered landscapes and have been shown to be sensitive to the effects of both urbanization (Russo and Ancillotto, 2015) and agriculture (Park, 2014). Bats are thus exposed to multiple sources of anthropogenic pollution yet there is a major scarcity of bat ecotoxicological studies. In fact, Zukal et al. (2015) specifically highlighted the low number of articles showing direct adverse effects of metals on bats, and provided various reasons why bats are ideal bioindicators of heavy metal pollution. For instance, the rapid metabolic processes of insect-eating bats requires that they consume an high quantity of prey per night, nearly equivalent to their body mass (Kurta et al., 1989). Furthermore, they are a high trophic level component of the ecosystem, which increases their susceptibility to pollutant accumulation through their diet (Hernout et al., 2013). Bioaccumulation of metals, for instance, is characteristically more prominent in carnivorous than in herbivorous small mammals (Alleva et al., 2006; Hamers et al., 2006).

For many insect-eating bats, rivers and streams, are important foraging habitats (Biscardi et al., 2007; Grindal et al., 1999; Racey et al., 1998; Warren et al., 2000). Insect-eating bats may thus be particularly vulnerable to water pollution because riparian vegetation and the emergent aquatic insects upon which bats feed are in direct contact with the polluted water (Walsh and Harris, 1996). In addition to foraging at rivers and other water bodies, bats may also ingest pollutants by drinking water at these contaminated sites. Water quality effects on bat activity are however, often species specific (Ciechanowski, 2002, Korine et al., 2015). For instance, Kalcounis-Rueppell et al. (2007) found that the activity of *Eptesicus fuscus* was lower downstream than upstream of a sewage output in North Carolina, USA. In contrast, *Perimyotis subflavus,* a species that specializes in riparian habitats (Ford et al., 2005), was more abundant at downstream sites (Kalcounis-Rueppell et al., 2007).

Thus, poor water quality does not affect all bat species negatively. In fact, some bat species may, in the short term, benefit from the proliferation of prey insects in polluted water. Vaughan et al. (1996) also investigated differences in bat activity above and below sewage outputs and found that certain species such as *Pipistrellus pipistrellus* were most active upstream of a pollution source, whereas others such as *Myotis* species concentrated their feeding activity downstream of the pollution source. At Irish rivers, Abbott et al. (2009) found that *P. pygmaeus* was significantly more active at downstream sites, while *M. daubentonii* was less active. Although the diet of the bats were not investigated, the trend in bat species abundances at upstream versus downstream sites was attributed to possible preferential feeding on the specific insect prey found at the sites. Racey et al. (1998) compared two rivers with differing water qualities and established that a river with inferior water quality could support bat activity and insect density as great as a healthy river. In Durban, South Africa, Naidoo et al. (2011) found greater bat diversity and activity at a polluted river than an unpolluted river, but the proportion of insect orders in the diet of bat species showed no correlation with the proportion of insect orders captured at the rivers.

Thus, there is evidence that bats, specifically riparian specialists, either exploit the high availability of pollution-tolerant insects in river habitats polluted by WWTWs, or avoid these habitats (Park and Cristinacce, 2006; Racey et al., 1998; Vaughan et al., 1996). Few studies, however, have investigated if bats that utilise polluted sites suffer potential physiological effects. Thus, while it appears that certain bat species benefit in the short-term, there may be long-term consequences for their health. For example, Pilosof et al. (2013), found that exposure to sewage water activates the immune response in *P. kuhlii*, causing a decrease in neutrophil levels and an increase in lymphocytes. Lilley et al. (2013) found that, although organic tin compounds did not increase

oxidative damage, it was associated with an immune system response of decreased complement activity in *M. daubentonii*. In addition, Pikula et al. (2010) showed that vespertilionid bats foraging over aquatic habitats are exposed to toxic heavy metals in the Czech Republic. A biological response was also found in these bats, where the metal-binding protein, metallothionein, was higher in aquatic-insect-foraging bats than in those foraging in terrestrial or terrestrial/ aquatic habitat. It is thus likely that bat species chronically exposed to pollutants may be experiencing sub-lethal physiological effects.

1.5. Neoromicia nana - *the urban adapter*

Studies in southern Africa have recently begun to elucidate the effects of urban water pollution on bat diversity and activity (Naidoo et al., 2011). At a wastewater-polluted river in Durban, the majority (approximately 41%) of bats recorded at the polluted river was represented by the banana bat, *Neoromicia nana* (Family Vespertilionidae) (Naidoo et al., 2011). *N. nana* is a small (3 - 4 grams), insect-eating bat that commonly occurs in forest and riparian habitats throughout sub-Saharan Africa (Monadjem et al., 2010). Although *N. nana* typically roosts in rolled-up banana leaves (LaVal and LaVal, 1977), it has also been found to roost in anthropogenically provided spaces such as thatched roofs of houses (Monadjem and Fahr, 2007; O'Shea, 1980) and curled leaves of strelitzia (*Strelitzia caudate and S. nicolaii*) and banana trees (Musa and Ensete spp.) planted in private gardens (M.C. Schoeman, unpubl. data).

N. nana's utilization of urban roosts and its high abundance along polluted urban rivers indicates that it is an urban adapter; i.e. a species that profits from resources provided by humans (Jung and Kalko, 2011). The small size and the fact that it is an urban adapter suggests that *N. nana* should exploit the increased availability of small chironomid midges at wastewater-polluted sites. Furthermore, chironomid activity is at its peak during the early evening (Broza et al., 2003), which correlates with the foraging period of *N. nana*. The toxic effects of pollution should thus be evident in *N. nana*, making this species an ideal model predator to assess the impact of wastewater pollutants.

1.6. Outline of thesis

There have been no studies to investigate whether African bats utilize WWTWs as foraging grounds, and no recorded studies to investigate the potential physiological impacts from foraging at such sites. The purpose of this thesis was to investigate the impact of WWTWs on foraging ecology and multiple tiers of physiology (haematology and genotoxicity, detoxification organs and reproduction) in an urban adapter, the banana bat (*Neoromicia nana*) in KwaZulu-Natal, South Africa. In obtaining an overall representation of the risk of wastewater impact on *N. nana*, I ask the following questions and test the following predictions:

1. Does wastewater pollution affect foraging ecology and metal concentrations in *N. nana* at sites receiving and not receiving wastewater effluent along three urban rivers in Durban, South Africa (Chapter 2)? I predicted that the abundance of the pollution-tolerant chironomid midges associated with wastewater and the relative abundance and feeding activity of *N. nana* would be higher at wastewater-polluted sites (WWTW tank and downstream sites) than at sites situated upstream of the wastewater pollution. If so, I further predicted that there should be a significant correlation between the abundance of pollution-tolerant insects at wastewater-polluted sites and in the diet of *N. nana* at the sites. I also predicted that metal content at the sites and in tissues of bats should be higher at wastewater-polluted sites (WWTW tank and downstream sites) than at sites situated upstream of the wastewater pollution.

2. Does foraging at wastewater-polluted sites elicit sub-lethal haematological and genotoxic responses in *N. nana* (Chapter 3)? Measures of haematological/genotoxic damage which are commonly used in routine assessments of ecotoxicological responses to environmental pollution were selected. I predicted that compared to bats foraging at unpolluted sites, *N. nana* foraging at WWTWs should have a greater extent of DNA damage, compromised total antioxidant capacity, higher levels of chromosomal aberration indicated by micronuclei formation, and altered blood oxygen capacity based on haematocrits.

3. How does pollutant exposure impact the detoxification organs, namely the liver and kidney of *N. nana* foraging at WWTWs (Chapter 4)? I predicted that organs of WWTW bats should reveal higher levels of toxic non-essential metals in liver and kidney tissue, based on SEM-EDS imaging to quantify metals and mineral nutrients. I also predicted that *N. nana* foraging at WWTWs should have a greater extent of histopathological lesions in the liver and kidney tissue, higher renalsomatic and hepatosomatic indices (characteristic of organ swelling due to metal

damage) and upregulated metal detoxification (metallothionein 1E) proteins compared to *N. nana* foraging at unpolluted sites.

4. Is the reproductive system of male *N. nana* foraging at WWTWs altered in the context of four hypotheses of EDC effects (Chapter 5)? I predicted that, compared to male *N. nana* foraging at unpolluted sites, *N. nana* foraging at WWTWs should have a lower concentration of plasma testosterone (the primary male sex hormone), reduced baculum morphometric parameters indicative of early-life exposure to pollutants, lower gonadosomatic indices (GSI) indicating whole organ effects on testes, and lower body condition as a general indication of male quality and fitness.

Finally, in Chapter 6, I synthesize the conclusions of the previous chapters, specifically within the framework of the main aims of the research. Factors contributing to differences in the foraging behaviour of *N. nana* at sites polluted and unpolluted by wastewater effluent, physiological responses, and the potential health and population effects in pollutant exposed *N. nana* are discussed. Implications of wastewater pollution for urban *N. nana* populations, bat communities and river ecosystems are explored. To conclude, recommendations for future studies are made.

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Chapter 2

Foraging at wastewater treatment works increases the potential for metal accumulation in an urban adapter, the banana bat (*Neoromicia nana*)

Foraging at wastewater treatment works increases the potential for metal accumulation in an urban adapter, the banana bat (*Neoromicia nana*)

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Wastewater treatment works (WWTWs) are known to provide profitable foraging areas for insectivorous bats in Europe and the New World because of their association with high abundance of pollution-tolerant midges (Diptera). However, bats that feed on these insects may also accumulate metal pollutants such as cadmium and copper in their tissues, with acute or chronic effects on their health. Using a time expansion bat detector, the activity (number of passes and feeding buzzes) of *Neoromicia nana* (family Vespertilionidae) was quantified at three WWTW points – upstream, the point of wastewater effluent discharge, and downstream – along three urban rivers in South Africa. In addition, metal concentrations in the kidney, liver and muscle tissue of *N. nana* caught at the sites were quantified. The diversity of aerial insects, sampled over the same period as the bat surveys, was measured using a black light trap and sweep-netting. Relative abundance and feeding activity of *N. nana* were higher at wastewater-polluted sites than at upstream sites. The most abundant insect order at wastewater-polluted sites and in the diet of resident bats was Diptera. Essential metals (copper, zinc and iron) were detected in all *N. nana* tissue samples, but the toxic metals cadmium, chromium and nickel were mostly present in tissue of bats at wastewater-polluted sites. Thus, although WWTWs provide an optimal food resource to bats in the short-term, it may pose serious long-term health risks for these top predators.

Key words: ***Neoromicia nana*, urban adapter, wastewater metal pollution, chironomid midge, diet.**

INTRODUCTION

A major anthropogenic disturbance within the urban landscape is river pollution from a range of inorganic and organic contaminants (Gleick 1998), such as chemical runoff from textile factories and sewage effluent (Sacks & Buckley 2004). These pollutants often have an adverse effect on animal biodiversity (Nedeau *et al.* 2003; Azrina *et al.* 2006; Vorosmarty *et al.* 2010) due to direct physiological effects on organisms or through modifications to the habitat or prey (Bridges & Semlitsch 2000). For example, metal pollutants taken up through the drinking of polluted water or the ingestion of contaminated prey can directly or indirectly affect the health of organisms, resulting in impaired physiology (Sánchez-Chardi *et al.* 2009), reproduction (Eeva *et al.* 2009) and behaviour (Mogren & Trumble 2010) or, in severe cases, mortality (Hoenerhoff & Williams 2004). Bioaccumulation of metals is characteristically more prominent in carnivorous small mammals than in herbivorous small mammals (Alleva *et al.* 2006; Hamers *et al.* 2006).

Insect-eating predators such as bats may be particularly vulnerable to organic and inorganic contaminants in water because riparian vegetation and the emergent aquatic insects upon which bats feed may be in direct contact with the polluted water (Walsh & Harris 1996). Aquatic insects such as chironomid midges (family Chironomidae) thrive in urban rivers polluted by wastewater works (Boonstra *et al.* 2009) because organic nutrients such as nitrogen (N) and phosphorous (P) promote eutrophication (Yount & Crossman 1970). At the same time, however, the tissues of these chironomid midges usually contain high concentrations of metal pollutants such as cadmium and copper, without showing adverse effects on survival (Krantzberg & Stokes 1990). Bats that feed on these midges may also accumulate metals in their tissues with acute or chronic effects on their health (Fritsch *et al.* 2010).

Wastewater treatment works (WWTWs) provide

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an ideal model to investigate the effect of river pollution on bats. WWTWs are ubiquitous in urban landscapes and are in constant operation. According to the Green Drop Report by the Department of Water Affairs (DWAF 2011), 56% of South Africa's WWTWs are in a poor to critical state, suggesting that treated effluent that is discharged into rivers by these WWTWs differ in their compliance with national chemical, microbial and physical standards. There is evidence that bats in Europe and North America, specifically riparian specialists, either exploit the high availability of pollution-tolerant insects in river habitats polluted by WWTWs, or avoid these habitats (Vaughan *et al.* 1996; Racey *et al.* 1998; Park & Cristinacce 2006). For example, *Myotis daubentonii*, one of three species of 'trawling bats' found in Europe, avoided nutrient-polluted sites and was more active upstream of sewage effluent discharges into Irish rivers (Abbott *et al.* 2009). Other riparian specialists such as *Perimyotis subflavus, Pipistrellus pygmaeus* and *Pipistrellus pipistrellus* species were more active at sites downstream of sewage outputs than upstream (Kalcounis- Rueppell *et al.* 2007; Abbott *et al.* 2009). These riparian specialists, unlike *M. daubentonii*, are better adapted to capturing smaller, airborne prey such as the pollution-tolerant midges at downstream sites (Fenton & Bogdanowicz 2002). However, small body size coupled with the fact that bats have relatively long life spans compared to similarly sized non-volant small mammals (Barclay & Harder 2003) means that these bats may have also a high risk of accumulating metals in organs (Walker *et al.* 2002). Although the lethal effects of large contaminant doses on bats have been documented (Clark *et al.* 1978), sublethal effects, such as metal-induced damage in target organs, endocrine disruption and modulation of the endocrine system have received far less attention, and often remain undetected in bats (Clark & Shore 2001).

The small (3–4 g), insect-eating bat *Neoromicia nana* (family Vespertilionidae) is known as the banana bat because it typically roosts in rolled-up banana leaves (Laval & Laval 1977), but it may also roost in thatched roofs of houses (O'Shea 1980; Monadjem & Fahr 2007). *N. nana* commonly occurs in forests and savanna, particularly in riparian habitats, throughout sub-Saharan Africa (Monadjem & Reside 2008; Monadjem *et al.* 2010a). *N. nana* has an intermediate bandwidth, frequency-modulated low duty-cycle echolocation call and short, broad wings with low wing loading (Monadjem *et al.* 2010b), suggesting that it forages near the edge of vegetation (Schnitzler & Kalko 2001). Moreover, *N. nana* appears to be an urban adapter (*sensu* Jung & Kalko 2011), i.e. it is able to exploit resources provided by humans. For example, in the subtropical city of Durban in South Africa, *N. nana* roosts in the curled leaves of strelitzia (*Strelitzia caudate* and *S. nicolaii*) and banana trees (*Musa* and *Ensete* spp.) planted in private gardens (M.C. Schoeman, unpubl. data) and occurs in large numbers along polluted rivers, feeding on small insects such as chironomid midges (Naidoo *et al.* 2011).

The aim of this study was to evaluate the influence of wastewater pollution on the foraging ecology and metal concentrations of *N. nana* at sites both receiving and not receiving wastewater effluent along three urban rivers in Durban, South Africa. We predicted that the abundance of the pollution-tolerant chironomid midges associated with wastewater and eutrophication (Marques *et al.* 1999), the relative abundance and feeding activity of *N. nana*, and metal content at the sites and in tissues of bats would be higher at wastewater-polluted sites (tank and downstream sites) than at sites situated upstream of the wastewater pollution.

METHODS

Study area

The study was conducted in the urban landscape of Durban, South Africa (S29°58'; E30°57'). There are approximately 32 WWTWs that operate within the Durban Metropolitan (Ceroi 1999). Three rivers that receive effluent from WWTWs with activated sludge tank systems were selected: Verulam Wastewater Works (S29°38.38; E31°03.49) situated on the Mdloti River (DWAF 2009), Kingsburgh Wastewater Works (S30°04.29; E30°51.26) situated on the Little Amanzimtoti River (Naidoo *et al.* 2002) and Umbilo Wastewater Works (S29°50.44; E30°53.31) situated on the Umbilo River (Lacko *et al.* 1999) (Fig. 1). According to the Department of Water Affair's Green Drop Report (DWAF 2011), Verulam and Umbilo had poor wastewater quality compliance, while Kingsburg's compliance was excellent. Study sites were situated (i) at least 3 km upstream of the point of effluent discharge into the river, (ii) at the sludge tanks in the wastewater treatment works and (iii) at least 3 km downstream of the point of wastewater effluent discharge. Upstream and downstream sites at each river had similar abiotic and

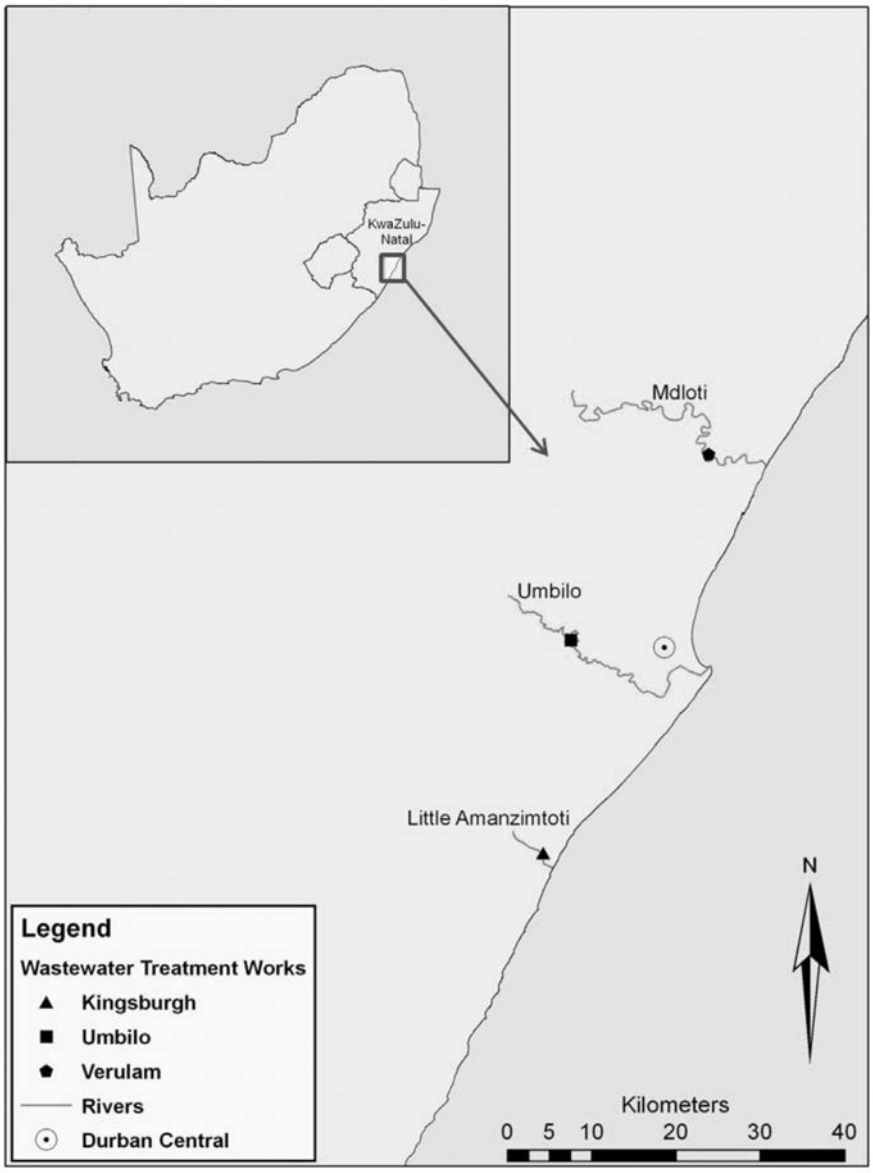

Fig. 1. Map of the study area in Durban, South Africa, showing the location of the Verulam, Kingsburgh and Umbilo Wastewater Works on the Mdloti, Little Amanzimtoti and Umbilo Rivers, respectively.

biotic features including distance between sites, width between banks (3–9 m), water surface clutter (Biscardi *et al.* 2007) and riparian vegetation (visual assessment).

Metal content at sites

Water samples were collected at sites upstream of the point of wastewater effluent discharge, at the sludge tanks of WWTWs, and at sites downstream of the point of wastewater effluent discharge. At each site, water was collected at sunset to correspond with the feeding emergence times of bats. Water was collected just below the surface in plastic bottles prepared with 2 ml, 65% concentrated nitric acid (Jackson *et al.* 2007). Three replicates were taken at each of the nine sites, resulting in a total of 27 samples. After allowing for overnight nitric acid digestion, the samples were filtered through Advantec GA-55 (47 mm) glass fibre filter membranes on a filtration pump, to remove

particulates. Ten ml of the filtrate was refrigerated until analysis for metal content.

Metal content, including cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), nickel (Ni) and zinc (Zn), was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Calibration standards of metal solutions were tested using the 'automated analysis control' function on WinLab32 ICP Continuous software (Perkin Elmer, U.S.A.). Although mercury (Hg) is also a toxic metal pollutant in rivers (Moeletsi *et al.* 2004), it was not tested for because detection of this hydride requires specialized equipment (cold vapour system; Henry & Miles 2001) which was not available to us.

Neoromicia nana *sampling*

Neoromicia nana was sampled with active (mist-nets) and passive (echolocation recordings) methods at each study site for three nights during winter (June/July 2009) and summer (March/April 2010). We randomly sampled the three sites along each river during each season to minimize the effects of daily climatic variation on bat activity. Because it was not possible to sample all the study sites at the same time, we sampled study sites during nights with comparable climatic conditions, specifically similar temperature and no rain. We passively monitored the relative abundance and feeding activity of *N. nana* by recording bat echolocation calls at each site from 18:30 and 18:00 until 22:30 and 21:00 in summer and winter, respectively. Echolocation calls were recorded using an Avisoft Ultrasound 116 Bat Detector (Avisoft Bioacoustics, Berlin, Germany) connected to a laptop computer (Hewlett Packard Pavilion 6210 notebook). At upstream and downstream sites, the recording equipment was set up alongside the river with the microphone positioned at a 45° angle to record bats flying directly above the river. At tank sites, the recording equipment was set up a few metres from the tank, with the microphone pointing at a 45° angle toward the tank. Batsound Pro-Sound Analysis software (version 3.31b, Pettersson Elektronik AB, Upsala, Sweden) was used to analyse the recorded echolocation calls. A sampling rate of 500 000 Hz (16 bits, mono) with a threshold of 16 was used. The dominant harmonic, i.e. peak frequency (PF) and the bandwidth (BW) from each recorded bat pass was measured using the power spectrum (size 1024), and duration (DU) of the call was measured from the oscillogram (Schoeman & Jacobs 2008). *N. nana* calls were identified by comparing PF and DU of calls with those of reference calls (Schoeman & Jacobs 2008; Monadjem *et al.* 2010b; Naidoo *et al.* 2011).

While recording bat activity, we captured *N. nana* with mist-nets which were set across the rivers and next to the tanks, far enough from the recording equipment to minimize disturbance. Nets were checked every 10–15 minutes. Captured bats were sexed and their life-stage (juvenile or adult) was determined from the presence of cartilaginous epiphyseal plates (Anthony 1988). We measured forearm length (to nearest 0.1 mm) with callipers, and body mass with a Pesola scale (to nearest 0.5 g). Species were identified using a taxonomic key (Monadjem *et al.* 2010a). Bat species other than *N. nana* were released where they were caught. *N. nana* individuals were held individually in cotton bags overnight to collect faecal pellets, and released the next day where they were caught, except *N. nana* collected during the summer sampling period, which were used to test metal content present in tissue (see below).

Relative abundance and feeding activity

We defined a bat pass as a series of echolocation calls made by one individual (Saunders & Barclay 1992). We quantified the relative abundance of *N. nana* with an acoustic activity index (AI) (Miller 2001):

$$\mathrm{AI} = \sum_{1}^{n} P ,$$

where n = number of 1 min intervals for sampling night and P = sum of presence counts (*N. nana* passes within a 1 min interval = 1 present count).

We quantified the feeding activity of *N. nana* at each site as the total number of feeding buzzes recorded (Fenton *et al.* 1977). Feeding buzzes consist of high pulse-repetition rates of echolocation pulses emitted by animalivorous bats as they capture prey.

Insect diversity

At each site we captured nocturnal insects with a 22 W black-light bucket trap (Black 1974) for the same period that mist-nets were set. Black-light traps effectively sample Diptera, Lepidoptera and Coleoptera (Nabli *et al.* 1999). The black-light trap was positioned along tanks or above water level near the river (~1 m above water, 1–3 m from the edge of the river), and at least 50 m away from

the mist-nets to prevent the light from affecting bat activity. In addition, we captured insects by sweep-netting (20 sweeps) along the edge of the river or tank, every hour from the start to the end of the sampling period per night. Insects collected from the sweeps and the light trap were pooled and identified to order using a taxonomic key (Scholtz & Holm 1985). Diptera were further analysed to obtain the abundance of chironomid midges. At least one individual from each collected order was mounted on a slide and used as a reference library for dietary analyses.

Neoromicia nana *dietary analysis*

To obtain representative samples of dietary breadth of *N. nana* at individual and population level, five faecal pellets from each bat (Whitaker *et al.* 1996) and a minimum of 20 pellets per site (Whitaker *et al.* 1999), respectively, were collected for dietary analyses. Faecal samples were individually teased apart in 70% alcohol. Remnants of insect exoskeletons were identified to order with the aid of a classification key (Scholtz & Holm 1985) and a reference collection of insects trapped at each site. The percentage of the total pellet volume comprising each order present was visually estimated following Whitaker (1988).

Metal content in Neoromicia nana *tissue*

Twenty-six adult *N. nana* captured at upstream ($n = 3$), tank ($n = 15$), and downstream ($n = 8$) sites along the Umbilo, Little Amanzimtoti and Mdloti rivers during summer (March/April 2010) were analysed for metal content. These bats were euthanased by decapitation, as approved by the University of KwaZulu-Natal Animal Ethics Committee (Reference: 086/12/Animal), and consistent with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (AVMA 2013). Bats were dissected for the collection of liver, kidney and pectoral muscle samples. The removed tissue samples were kept at –80°C until preparation for metal analysis. Samples were dried at 60°C for two days in an oven and then weighed to obtain the dry mass of the sample. Using a ratio of 45.5 ml HNO_3: 1 g tissue, the dried tissue samples were digested overnight in 65% concentrated nitric acid. To remove particulate matter, the digested samples were diluted with distilled water (1:2) and filtered through syringe filters with a diameter of 25 mm and pore size of 0.45 μ (PALL, Acrodisc). The liquid filtrate was kept for analysis.

Dry weight concentrations of Cu, Cr, Fe, Ni, Zn, Pb and Cd in liver, kidney and muscle samples were determined using ICP-OES (Perkin Elmer, Optima 5300 DV). The only metal in the tissue samples where all concentrations were below detection of the ICP-OES was Pb (DL = 0.0420 μg/g), which was therefore not presented in the results. The major limitation of ICP-OES is the high detection limit for metal quantification. A number of measured concentrations for Cd, Ni and Cr were also below their detection limits (DL = 0.0027 μg/g; 0.0150 μg/g; 0.0071 μg/g respectively). All metal concentrations below detection limits were assigned the value of the detection limit for the respective metal.

To test whether the recovery rates of metals were consistent across all sites, the same procedure used to prepare *N. nana* tissue samples for metal analysis, was performed on certified standard reference material (dried oyster tissue, SRM1566b) (National Institute of Standards and Technology). Recovery rates for the metals in the standard reference material (dried oyster tissue, SRM1566b) ranged from 67% to 150%, with the exception of Cd which had a poor recovery rate of 11%. However, metal concentrations between the three replicates of oyster samples analysed were consistent, with only 1% to 6% standard deviation between replicates. Thus, despite the wide range of recovery rates between metals, the trends across upstream, tank and downstream sites per metal should be consistent.

Statistical analyses

One-way ANOVAs and Tukey HSD *post hoc* tests were used to compare differences between upstream, tank and downstream sites for each of the metals at the rivers. Three-way ANOVAs were used to compare differences in *N. nana* relative abundance, *N. nana* feeding activity, total insect abundance, insect order richness and midge abundance among sites (upstream, tank and downstream) and rivers, and between seasons (summer and winter). The proportion of insect orders in the diet of *N. nana* bats was compared with the proportion of insect orders at the rivers using a Pearson's correlation. Statistical analyses could not be used to compare differences in tissue metal concentrations among upstream, tank and downstream sites because of low sample sizes ($n = 0$ or 1 for some sites). We conducted simple linear regressions to determine the extent to which metal concentrations measured in *N. nana* tissue (dependent vari-

able) are associated with metal concentrations in the water (independent variable). Assumptions of normality and equality of variance were tested using a one-sample Kolmogorov-Smirnov test and a Levene's test, respectively. If assumptions were not satisfied, non-parametric tests were run. All analyses were performed with SPSS 19.0, using alpha of 0.05.

RESULTS

Metal content in water

There were significant differences in metal content in water among downstream, tank and upstream sites (Fig. 2a–c), except in Pb at the Umbilo and Mdloti rivers (Fig. 2a,c), and in Ni at the Little Amanzimtoti River (Fig. 2b). Metal content was highest at tank sites, except Cu and Fe, which were highest upstream at the Little Amanzimtoti River (Fig. 2a–c).

Neoromicia nana relative abundance and feeding activity

Neoromicia nana emits low duty-cycle, frequency modulated echolocation calls (LD-FM). The PF of *N. nana* was 68.6 ± 2.0 kHz, with a BW of 14.1 ± 3.7 kHz and a DU of 4.6 ± 0.8 ms (mean ± S.D.; n = 10). Passive monitoring revealed 5361 *N. nana* call sequences: 668 were recorded at upstream sites, 2142 at tank sites, and 2551 at downstream sites. There was a significant difference in the relative abundance of *N. nana* among upstream, tank and downstream sites ($F_{(2,49)}$ = 84.424, P < 0.0005; Fig 3a). Tukey HSD *post hoc* tests showed that relative abundance was significantly higher at downstream than at tank and upstream sites (P < 0.0005). The lowest relative abundance was at the upstream sites (Fig. 3a). There was no significant difference in *N. nana* relative abundance among rivers, however there was a significant interaction effect between river and site ($F_{(4,49)}$ = 6.783, P < 0.0005), indicating that the difference in abundance among sites was not consistent among rivers. There were no significant seasonal differences in relative abundance.

Feeding activity (i.e. number of feeding buzzes) was significantly higher at wastewater-polluted sites than at upstream sites ($F_{(2,49)}$ = 10.315, P < 0.0005; Fig. 3b), but did not differ significantly between tank and downstream sites. There were also no significant seasonal differences in feeding activity. Feeding activity differed significantly among rivers, with the Little Amanzimtoti River having significantly lower numbers of feeding buzzes than the Umbilo and Mdloti rivers ($F_{(2,49)}$ = 9.438, P < 0.0005).

Insect abundance and richness of orders

The total number of insects captured during both sampling seasons was 3742 at the Umbilo River, 3209 at the Little Amanzimtoti River and 3513 at the Mdloti River. At all three rivers, the most prevalent order at the upstream sites was Coleoptera (45.8–69.8%) and at wastewater-polluted sites it was Diptera (41.2–73.1%; except for the Umbilo downstream site, which varied) (Table 1). Neuroptera, Dermaptera and Mantodea were rare (≤2%) and were pooled into one category classified as 'other'. Trichoptera and Ephemeroptera were more abundant at upstream sites than at wastewater-polluted sites (Table 1). More than 80% of Diptera caught at the tank and downstream sites comprised chironomid midges.

There was a significant difference in insect abundance and number of insect orders among the upstream, tank and downstream sites,and between seasons (all P < 0.05, Table 2). Tukey HSD *post hoc* tests showed that downstream sites had a significantly higher insect abundance and number of insect orders than tank sites (Table 2). The number of insect orders was also significantly higher at upstream sites than at tank sites. There were significantly higher abundances and number of insect orders in summer than in winter (Table 2). In addition, there were several significant interaction effects between sites, season and river for insect abundance (see Table 2). Mean abundance of midges per night (Fig. 4) was significantly higher at wastewater-polluted sites than at upstream sites, and at tank sites than at downstream sites (Table 2). Midge abundance was significantly higher in summer than in winter, and at the Little Amanzimtoti and Mdloti rivers than at the Umbilo River (Table 2). There were also significant interaction effects between site, season and river (Table 2).

Neoromicia nana diet

In total, 86 *N. nana* were captured: five individuals at upstream sites, 45 individuals at tank sites and 36 individuals at downstream sites. At least 20 faecal pellets were analysed for *N. nana* captured at the upstream, tank and downstream sites except at the Umbilo upstream site where no faecal pellets were collected from the single *N. nana* captured. At all tank and downstream sites (except downstream at Little Amanzimtoti in

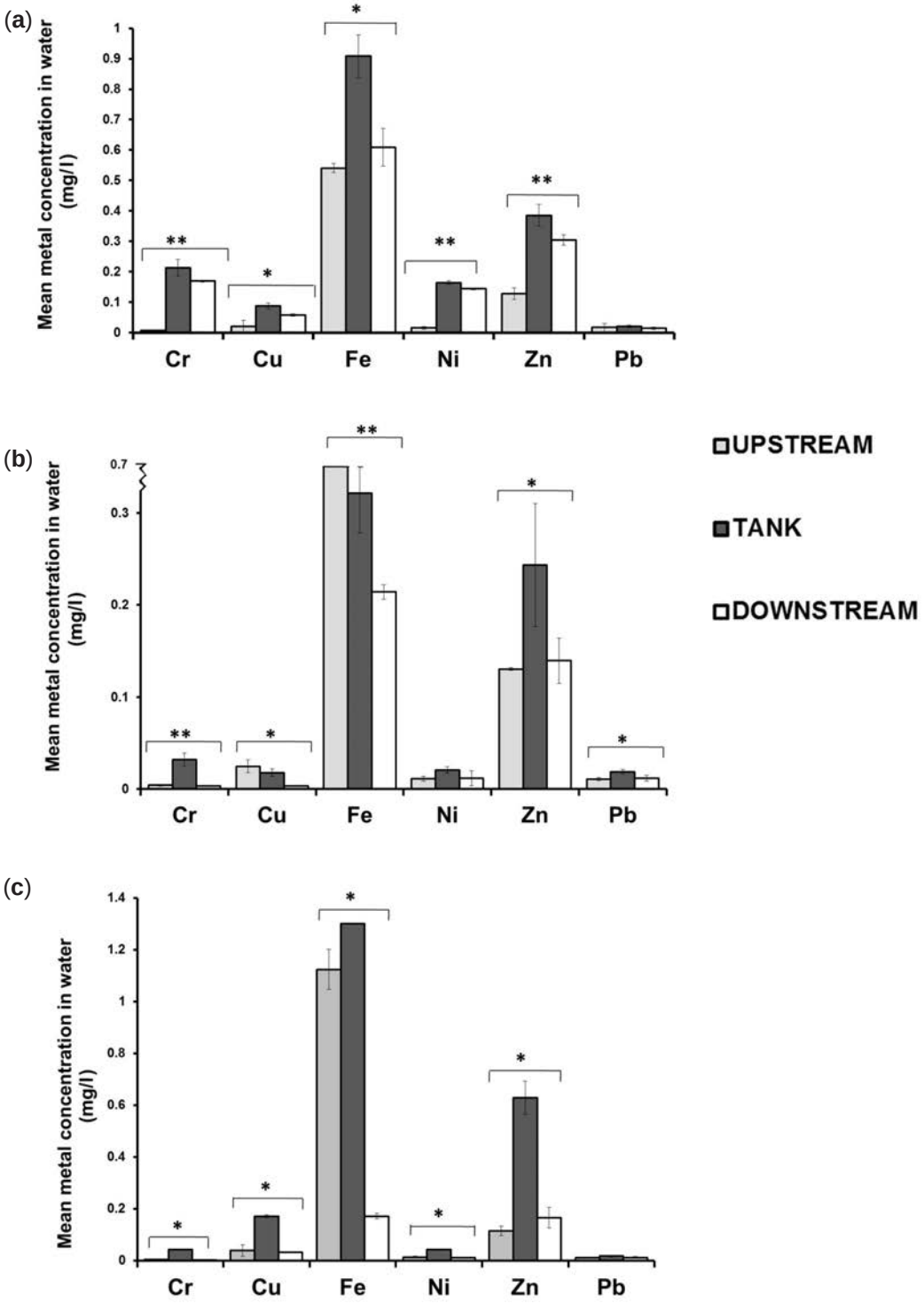

Fig. 2. Mean concentrations (mg/l) of chromium (Cr), copper (Cu), iron (Fe), nickel (Ni) and lead (Pb) at upstream, tank and downstream sites at the (**a**) Umbilo, (**b**) Little Amanzimtoti and (**c**) Mdloti rivers (* and ** indicate significant differences between the sites at the $P < 0.05$ and $P < 0.0005$ levels, respectively). Bars = ±S.E.

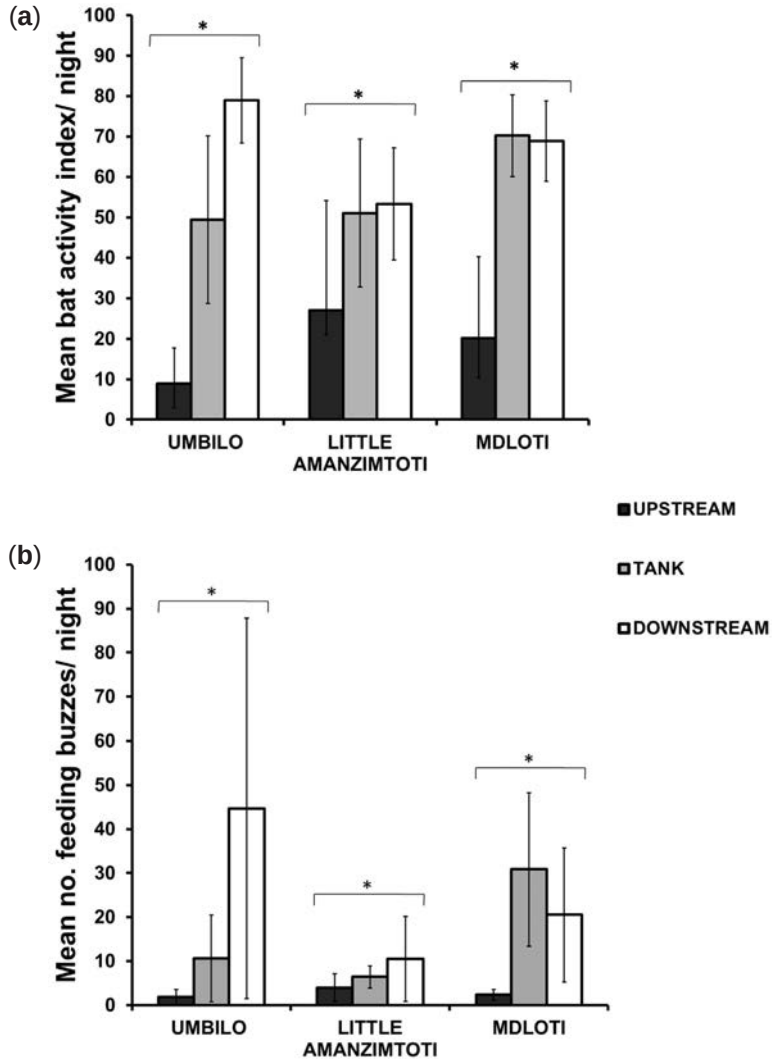

Fig. 3. Mean *Neoromicia nana* activity index per night (**a**) and number of feeding buzzes per night (**b**) at upstream, tank and downstream sites (* indicates significant differences between the sites at the $P < 0.001$ level). Bars = ±S.E.

winter), the insect order constituting the highest proportion (or second highest by ≤1%) in the diet of *N. nana* was Diptera (Table 3). At upstream sites, Coleoptera was the insect order constituting the highest proportion (or second highest by ≤4%) in the diet of *N. nana* (Table 3).

The proportions of insect orders in the diet of *N. nana* were significantly correlated to the proportions of insect orders captured at the majority of wastewater-polluted sites: the tank sites at Umbilo and Mdloti in summer, and Little Amanzimtoti in summer and winter, the downstream sites at Mdloti in both seasons, Umbilo in winter and the Little Amanzimtoti in summer (all $P < 0.05$, all $r < 0.1$) (Table 3). This indicates that at wastewater-polluted sites, *N. nana* generally fed opportunistically. Among the upstream sites, only Mdloti River in summer had proportions of insect orders significantly correlated to those in the diet of *N. nana,* suggesting that *N. nana* fed selectively at the other upstream sites.

Metal content in *Neoromicia nana* tissue

Cu, Zn and Fe were detected in all the tissue samples (raw data are available in the online supplement). Conversely, Cd, Cr and Niwere detected in tissue collected from only wastewater-polluted sites (except one occurrence

Table 1. Mean (±S.D.) percentage abundance (per night) of the insect orders Coleoptera (Col), Diptera (Dip), Lepidoptera (Lep), Hemiptera (Hem), Hymenoptera (Hym), Trichoptera (Trich) and Ephemeroptera (Ephem) captured at upstream (U), tank (T) and downstream (D) sites at the Umbilo, Little Amanzimtoti and Mdloti rivers during winter (W) and summer (S) in 2009 and 2010 (number of individuals ± S.D. in brackets).

River	Site	Season	% Insect order (number of individuals)							
			Col	Dipt	Lep	Hem	Hym	Trich	Ephem	Other
Umbilo	U	S	69.8 ± 33.8 (166.3 ± 80.5)	9.5 ± 10.1 (22.7 ± 24.0)	1.6 ± 1.0 (3.7 ± 2.5)	6.4 ± 3.6 (15.3 ± 8.5)	11.3 ± 2.1 (27 ± 5.0)	0.5 ± 0.3 (1.3 ± 0.6)	0.7 ± 0.6 (1.7 ± 1.5)	0.1 ± 0.3 (0.3 ± 0.6)
		W	45.8 ± 50.8 (27.0 ± 30.0)	12.4 ± 7.1 (7.3 ± 4.2)	2.9 ± 3.6 (1.7 ± 2.1)	34.4 ± 31.9 (20.3 ± 18.8)	1.2 ± 2.0 (0.7 ± 1.2)	2.2 ± 1.0 (1.3 ± 0.6)	1.2 ± 0.1 (0.7 ± 1.2)	0.0 (0.0)
	T	S	26.5 ± 3.7 (57.3 ± 8.0)	44.0 ± 12.1 (95.3 ± 26.3)	4.9 ± 3.1 (10.7 ± 6.8)	19.1 ± 5.8 (41.3 ± 12.6)	4.5 ± 1.5 (9.7 ± 3.2)	0.0 (0.0)	0.0 (0.0)	1.1 ± 1.8 (2.3 ± 4.0)
		W	19.9 ± 24.8 (17 ± 21.2)	73.1 ± 17.3 (62.5 ± 14.8)	2.3 ± 1.6 (2.0 ± 1.4)	4.1 ± 5.7 (3.5 ± 4.9)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.6 ± 0.8 (0.5 ± 0.7)
	D	S	44.1 ± 7.9 (269 ± 48.4)	16.1 ± 1.4 (98.3 ± 8.4)	1.3 ± 0.4 (8 ± 2.6)	35.6 ± 5.7 (217.3 ± 34.6)	0.8 ± 0.2 (4.7 ± 1.2)	0.1 ± 0.1 (0.7 ± 0.6)	0.0 (0.3 ± 0.6)	1.9 ± 1.7 (11.3 ± 10.4)
		W	27.6 ± 3.9 (20.0 ± 2.8)	58.6 ± 6.8 (42.5 ± 4.9)	3.4 ± 1.0 (2.5 ± 0.7)	9.0 ± 4.8 (6.5 ± 3.5)	1.4 ± 1.9 (1.0 ± 1.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Little Amanzimtoti	U	S	57.7 ± 14.5 (189.3 ± 47.6)	10.9 ± 7.8 (35.7 ± 25.6)	6.0 ± 1.2 (19.7 ± 4.0)	12.4 ± 6.0 (40.7 ± 19.6)	8.6 ± 5.2 (28.3 ± 17.2)	2.1 ± 1.1 (7.0 ± 3.5)	0.7 ± 1.0 (2.3 ± 3.2)	1.6 ± 0.7 (5.3 ± 2.3)
		W	46.0 (29.0)	19.0 (12.0)	4.8 (3.0)	12.7 (8.0)	14.3 (9.0)	3.2 (2.0)	0.0 (0.0)	0.0 (0.0)
	T	S	29.4 ± 14.2 (77.3 ± 37.3)	52.8 ± 7.5 (138.7 ± 19.7)	2.0 ± 0.8 (5.3 ± 2.1)	11.9 ± 4.1 (31.3 ± 10.8)	3.2 ± 1.7 (8.3 ± 4.5)	0.1 ± 0.4 (0.3 ± 0.6)	0.3 ± 0.2 (0.7 ± 0.6)	0.3 ± 0.2 (0.7 ± 0.6)
		W	39.2 ± 10.3 (32.7 ± 8.6)	43.2 ± 7.9 (36 ± 6.6)	2.8 ± 0.7 (2.3 ± 0.6)	14.0 ± 5.4 (11.7 ± 4.5)	0.8 ± 1.4 (0.7 ± 1.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	D	S	20.1 ± 12.9 (50 ± 32.1)	47.2 ± 4.5 (117.5 ± 11.2)	5.1 ± 2.0 (12.7 ± 4.9)	12.0 ± 2.4 (30.0 ± 6)	14.8 ± 4.6 (37 ± 11.5)	0.4 ± 0.5 (1.0 ± 1.0)	0.0 (0.0)	0.4 ± 0.4 (1.0 ± 1.0)
		W	21.0 ± 6.0 (26.3 ± 7.5)	52.1 ± 16.4 (65.3 ± 20.5)	3.8 ± 1.0 (4.7 ± 1.2)	12.2 ± 1.2 (15.3 ± 3.5)	10.1 ± 4.0 (12.7 ± 5)	0.2 ± 2.2 (0.3 ± 0.6)	0.0 (0.0)	0.6 ± 0.5 (0.7 ± 0.6)
Mdloti	U	S	48.1 ± 4.0 (200.3 ± 16.6)	4.1 ± 3.0 (17.0 ± 12.3)	6.9 ± 2.4 (28.7 ± 9.9)	31.8 ± 9.9 (132.3 ± 41.2)	1.4 ± 0.7 (5.7 ± 3.1)	6.1 ± 1.2 (25.3 ± 9.3)	0.5 ± 0.5 (2.0 ± 2.0)	1.1 ± 0.5 (4.7 ± 2.1)
		W	46.9 ± 6.2 (26.5 ± 3.5)	11.5 ± 3.7 (6.5 ± 2.1)	11.5 ± 3.7 (6.5 ± 2.1)	28.3 ± 5.0 (16.0 ± 2.8)	0.9 ± 1.2 (0.5 ± 0.7)	0.9 ± 0.4 (0.5 ± 0.7)	0.0 (0.0)	0.0 (0.0)
	T	S	31.9 ± 6.6 (94.0 ± 19.3)	55.8 ± 3.7 (164.3 ± 10.8)	2.5 ± 1.4 (7.3 ± 4.0)	6.7 ± 3.4 (19.7 ± 10.0)	1.5 ± 0.7 (4.3 ± 2.1)	0.2 ± 0.7 (0.7 ± 1.2)	0.0 (0.0)	1.5 ± 1.5 (4.3 ± 4.5)
		W	37.2 ± 7.7 (47.3 ± 9.8)	55.8 ± 7.5 (71.0 ± 9.5)	2.1 ± 0.5 (2.7 ± 0.6)	3.9 ± 1.3 (5.0 ± 1.7)	1.0 ± 0.5 (1.3 ± 0.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	D	S	28.1 ± 7.2 (64.0 ± 16.5)	41.2 ± 5.7 (93.7 ± 12.9)	10.5 ± 2.9 (24.0 ± 6.6)	9.2 ± 4.0 (21 ± 9.2)	7.3 ± 4.5 (16.7 ± 10.3)	1.0 ± 0 (2.3 ± 1.5)	0.0 (0.0)	2.6 ± 1.6 (6.0 ± 3.6)
		W	17.8 ± 8.9 (12.0 ± 6)	65.5 ± 19.7 (44.3 ± 13.3)	7.4 ± 2.5 (5.0 ± 1.7)	7.8 ± 5.2 (5.3 ± 3.5)	1.5 ± 1.5 (1.0 ± 1)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Table 2. Significant ($P < 0.05$) results for three-way ANOVAs and Tukey *post hoc* tests for insect abundance, number of insect orders and midge abundance among sites (upstream (U), tank (T), downstream (D)) and rivers (Umbilo (Umb), Little Amanzimtoti (L), Mdloti (M)), and between seasons (summer (S), winter (W)).

Variable	Effect	Significant three-way ANOVAs			Significant *post hoc* Tukey tests
		F	d.f.	*P*	
Insect abundance	Site	4.818	2,46	0.015	D>T
	Season	313.867	1,47	<0.0005	S>W
	Site × season	8.462	2,46	0.001	
	Site × river	16.911	4,46	<0.0005	
	River × season	4.038	2,46	0.028	
	Site × season × river	18.062	4,46	<0.0005	
No. of insect orders	Site	10.627	2,46	<0.0005	U>T, D>T
	Season	64.234	1,47	<0.0005	S>W
Midge abundance	Site	156.086	2,49	<0.0005	T>U, D>U, T>D
	Season	143.973	1,49	<0.0005	S > W
	River	10.372	2,49	<0.0005	L>Umb, M>Umb
	Site × season	36.468	2,49	<0.0005	
	Site × river	10.540	4,49	<0.0005	

of Cd at an upstream site) (Table 4). At all three rivers, the maximum Cd concentration at wastewater-polluted sites in the kidney, liver and muscle tissue was higher than (or equal to) the detection limit. Only upstream sites had Cd concentrations below detection and were therefore assigned the value of the detection limit of Cd (see ranges, Table 4). Although Cr and Ni were below detection in liver tissue, Cr was detected in one downstream kidney sample and in three muscle samples from the tank site. Ni was detected in kidney tissue sampled at the wastewater-polluted sites of the Mdloti River, and in muscle tissue samples from the wastewater-polluted sites of the Umbilo River (Table 4). There was a positive significant relationship between the metals quantified in the kidney and the metals in the water ($R^2 = 0.662$, $P < 0.0005$; $F = 48.974$; Fig. 5). However,

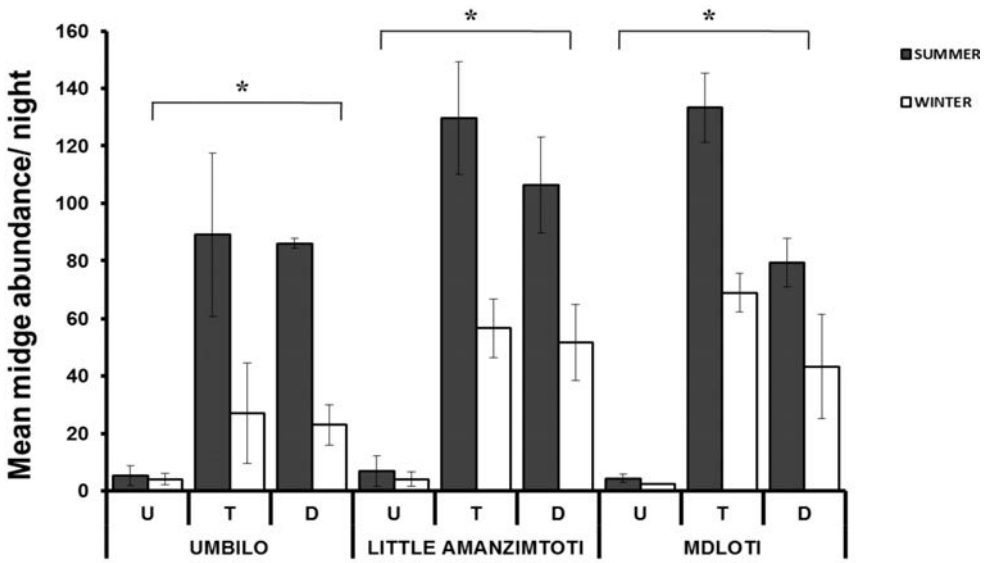

Fig. 4. Mean midge abundance per night at upstream (U), tank (T) and downstream (D) sites at the Umbilo, Little Amanzimtoti and Mdloti rivers during winter (2009) and summer (2010) (* indicates significant differences between the sites at the $P < 0.001$ level). Bars = ±S.E.

Table 3. Mean (±S.D.) percentage volume of the insect orders Coleoptera (Col), Diptera (Dip), Hymenoptera (Hym), Hemiptera (Hem), Lepidoptera (Lep) and Trichoptera (Trich) in the diet of *Neoromicia nana* captured at upstream (U), tank (T) and downstream (D) sites at the Umbilo, Little Amanzimtoti and Mdloti rivers during winter (W) and summer (S).

River	Site	Season	No. of pellets	Insect order (% volume diet composition)					
				Col	Dipt	Lep	Hem	Hym	Trich
Umbilo	T	S	15	14.0 ± 7.6	61.0 ± 5.3	9.3 ± 3.8	11.7 ± 10	2.7 ± 2.3	1.3 ± 2.3
		W	10	35.5 ± 17.7	35.0 ± 21.2	8.0 ± 4.2	8.5 ± 0.7	13.0 ± 1.4	0.0
	D	S	5	37.0 ± 2.7	46.0 ± 8.2	9.0 ± 5.5	6.0 ± 8.2	2.0 ± 2.7	0.0
		W	15	21.7 ± 24.3	50.0 ± 25.7	12.7 ± 10	8.0 ± 9.2	7.7 ± 6.8	0.0
Little Amanzimtoti	U	S	5	53.0 ± 2.7	4.0 ± 5.5	20.0 ± 0	10.0 ± 6.1	13.0 ± 6.7	0.0
		W	10	24.0 ± 9.9	28.0 ± 7.1	20.5 ± 12	14.0 ± 7.1	9.5 ± 3.5	4.0 ± 5.7
	T	S	20	35.3 ± 12.7	46.8 ± 10.3	6.0 ± 1.8	8.5 ± 7.2	3.5 ± 4.7	0.0
		W	15	40.0 ± 29.9	39.0 ± 28	9.3 ± 4	9.0 ± 4.4	2.7 ± 3.1	0.0
	D	S	5	12.0 ± 2.7	60.0 ± 12.2	7.0 ± 2.7	7.0 ± 6.7	14.0 ± 13.4	0.0
		W	20	32.3 ± 8.5	13.0 ± 10.1	27.5 ± 5.8	25.8 ± 12.7	1.5 ± 3.0	0.0
Mdloti	U	S	10	41.5 ± 23.3	2.0 ± 2.8	7.0 ± 7.1	32.5 ± 0.7	15.0 ± 15.6	2.0 ± 2.8
	T	S	20	22.3 ± 15.4	52.8 ± 33.3	7.3 ± 4.9	15.8 ± 14.9	2.0 ± 4.0	0.0
		W	40	11.9 ± 11.2	57.5 ± 15.4	3.8 ± 2.9	23.9 ± 11.7	3.0 ± 3.3	0.0
	D	S	20	23.8 ± 6.9	45.8 ± 10.4	7.8 ± 2.1	13.3 ± 4.6	9.5 ± 7.5	0.0
		W	43	22.4 ± 15.4	61.3 ± 16.7	1.5 ± 1.6	14.4 ± 8.0	0.0	0.33 ± 1

there were no significant relationships between metal in liver and muscle tissue and metal in the water (all $P > 0.05$).

DISCUSSION

As we predicted, the relative abundance and number of feeding buzzes of *N. nana* were highest at wastewater-polluted sites and lowest at sites located upstream of the wastewater pollution, presumably due to the higher abundance of insects, specifically Diptera, present at the wastewater-polluted sites. Diptera also comprised the largest proportion in *N. nana*'s diet at wastewater-polluted sites. This corresponds with previous studies, where high abundances and feeding rates of riparian specialist bats were recorded below sewage outfall, compared to unpolluted upstream sites (Vaughan *et al.* 1996; Racey *et al.* 1998; Kalcounis-Rueppell *et al.* 2007; Abbott *et al*. 2009). The significant correlations between insects captured and diet composition at most polluted sites suggests that here *N. nana* fed opportunistically on the abundant prey, specifically small dipterans. In contrast, the non-significant correlations between insects in the diet and those captured at most upstream sites (except at Mdloti River during summer) suggests that *N. nana* tended to forage selectively on insects from different orders, in particular moths (Lepidoptera), beetles (Coleoptera) and bugs (Hemiptera).

These trends suggest that the foraging ecology of *N. nana* might conform to predictions from optimal foraging theory where prey are ranked by the predator according to their profitability (MacArthur & Pianka 1966). Moths, bugs and beetles are energetically probably more profitable to bats than small midges, hence they are an important component of the diet of many insectivorous bat species (Monadjem *et al.* 2010b). This is also the case for *N. nana* at unpolluted sites in southern Africa (Fenton *et al.* 1977; Schoeman & Jacobs 2011). Thus, *N. nana* may not be foraging randomly, but instead is adapted to optimally exploit the high abundance of swarming pollution-tolerant midges at wastewater-polluted

Table 4. Mean (± S.D.) and range of cadmium, chromium and nickel concentrations (µg/g dry weight), and number of samples below detection limit (BDL) in the kidney, liver and muscle of *Neoromicia nana* at upstream, tank and downstream sites of the Umbilo, Little Amanzimtoti and Mdloti rivers.

		Cadmium			Chromium			Nickel		
		Mean ± S.D.	Range	BDL	Mean ± S.D.	Range	BDL	Mean ± S.D.	Range	BDL
Umbilo										
Upstream (*n* = 0)	–	–	–	–	–	–	–	–	–	–
Tank (*n* = 3)	Kidney	2.639 ± 1.582	0.819–3.686	0	0.007	0.007–0.007	3	0.015	0.015–0.015	3
	Liver	1.866 ± 1.290	0.410–2.867	0	0.007	0.007–0.007	3	0.015	0.015–0.015	3
	Muscle	0.047 ± 0.077	0.003–0.137	2	0.007	0.007–0.007	3	0.056 ± 0.070	0.015–0.137	2
Downstream (*n* = 1)	Kidney	1.775	–	0	0.007	–	1	0.015	–	1
	Liver	0.546	–	0	0.007	–	1	0.015	–	1
	Muscle	0.003	–	0	0.007	–	1	1.229	–	0
Little Amanzimtoti										
Upstream (*n* = 1)	Kidney	0.003	–	1	0.007	–	1	0.015	–	1
	Liver	0.003	–	1	0.007	–	1	0.015	–	1
	Muscle	0.003	–	1	0.007	–	1	0.015	–	1
Tank (*n* = 6)	Kidney	0.910 ± 0.948	0.003–2.594	1	0.007	–	6	0.015	–	6
	Liver	0.071 ± 0.166	0.003–0.410	5	0.007	–	6	0.015	–	6
	Muscle	0.025 ± 0.055	0.003–0.137	5	0.007	0.007–0.007	6	0.015	0.015–0.015	6
Downstream (*n* = 1)	Kidney	0.273	–	0	0.007	–	1	0.015	–	1
	Liver	0.003	–	1	0.007	–	1	0.015	–	1
	Muscle	0.003	–	1	0.007	–	1	0.015	–	1
Mdloti										
Upstream (*n* = 2)	Kidney	0.070 ± 0.097	0.003–0.137	1	0.007	0.007–0.007	2	0.015	0.015–0.015	2
	Liver	0.003	0.003–0.003	2	0.007	0.007–0.007	2	0.015	0.015–0.015	2
	Muscle	0.003	0.003–0.003	2	0.007	0.007–0.007	2	0.015	0.015–0.015	2
Tank (*n* = 6)	Kidney	0.614 ± 0.645	0.137–0.956	0	0.007	0.007–0.007	6	1.375 ± 3.272	0.015–8.054	4
	Liver	0.070 ± 0.113	0.003–0.273	4	0.007	0.007–0.007	6	0.015	0.015–0.015	4
	Muscle	0.003	0.003–0.003	6	1.209 ± 2.313	0.007–0.956	3	0.015	0.015–0.015	6
Downstream (*n* = 6)	Kidney	0.228 ± 0.111	0.137–0.410	0	0.120 ± 0.276	0.007–0.683	5	3.289 ± 8.018	0.015–19.656	5
	Liver	0.003	0.003–0.003	6	0.007	0.007–0.007	6	0.015	0.015–0.015	6
	Muscle	0.071 ± 0.166	0.003–0.410	5	0.007	0.007–0.007	6	0.015	0.015–0.015	6

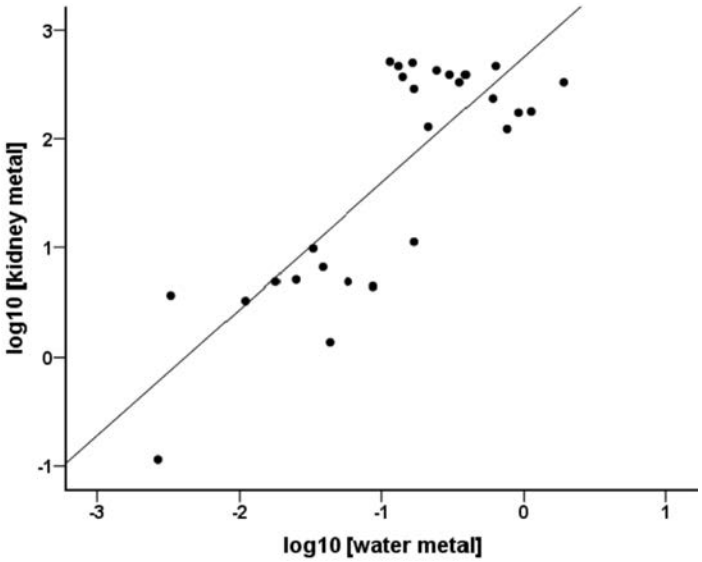

Fig. 5. Relationship between the concentrations of metals in the kidney and the concentrations of the metals in the water (regression equation: $y = 1.158x + 2.753$) in *Neoromicia nana* at upstream, tank and downstream sites of the Umbilo, Little Amanzimtoti and Mdloti rivers.

sites in urban landscapes. However, ultimately less profitable items should be eaten only when the energy gained from eating them exceeds the energy gained from rejecting them and finding more profitable prey (Jones 1990), mediated by prey abundance. To test this hypothesis, future studies should quantify the differences in energy returns for bats between catching many small midges flying in swarms, and fewer, more widely dispersed, large fast-flying insects.

Moreover, molecular studies of bat diet may (e.g. Razgour *et al.* 2011) or may not (e.g. Alberdi *et al.* 2012) support the course-grained results of microscopic dietary analyses. Nevertheless, molecular studies provide a high resolution of prey identification – often to species level – which provide insights into fine-scale patterns in resource use. Furthermore, all insect capture methods are biased towards catching insects of a particular order, size or mobility (Muirhead-Thomson 1991). Thus, analyses investigating the relationships between the diet of *N. nana* and prey availability using molecular dietary data and prey data from additional capturing methods, such as suction or sticky insect traps or different lights, may yield different results to those we report here.

Pollution-tolerant insects such as chironomid midges (Postma *et al.* 1995) constituted more than 80% of all Diptera at wastewater-polluted sites. In contrast, pollution-sensitive invertebrates from orders such as Ephemeroptera and Trichoptera (Dinakaran & Anbalagan 2007) were less abundant here than at upstream sites; thus insect order richness was low at polluted sites. At sites polluted with industrial discharge chironomid midge larvae have shown morphological deformities such as head capsule and mouthpart asymmetry, yet no adverse effects on their survival and growth to adults was shown (Al-Shami *et al.* 2010). Similarly, metal-adapted genetic strains of chironomid midges have been found at sites downstream of pollution (Groenendijk *et al.* 1998). Although not tested in this study, the tissue of chironomid midges at metal-contaminated sites usually contain high concentrations of metals (Krantzberg & Stokes 1990), which are transferred from substrate to larvae to adult (Reinhold *et al.* 1999). This puts those bats that opportunistically feed on the large swarms of chironomid midges at polluted sites at risk of accumulating metals themselves.

The essential metals Cu, Zn and Fe were detected in all tissue samples from *N. nana* foraging at upstream and wastewater-polluted sites. However, the non-essential metals Cd, Ni and Cr were detected only in *N. nana* foraging at wastewater-polluted sites (except one upstream occurrence of Cd). While Cu, Zn and Fe are essential for normal cellular processes and bodily function, Cd, Cr and Ni are considered harmful to organisms (Hoffman *et al.* 2001). We found a signif-

icantly positive relationship between concentrations of metals in the kidney tissue and metals in the water. This is an important result because the kidney is the main storage site of toxic metals including Cd (Hunter & Johnson 1982). In addition, the liver and kidney actively regulate essential metals, thus concentrations of toxic metals in these tissues reflect exposure and accumulation of those metals for a prolonged period (McGeer *et al.* 2000). This suggests that there is potential for transfer of metals through the food chain.

Cd was present in kidney, muscle and liver. Cd is known to have deleterious effects on health (Henson & Chedrese 2004; Burger 2008). It is one of the metals of most concern to wildlife, and is a teratogen, carcinogen and possible mutagen (Burger 2008). It has also been recently recognized as an endocrine-disrupting chemical (EDC), reported to mimic the effects of oestrogen in the body (Henson & Chedrese 2004). The concentrations obtained for tissue Cd in wastewater-polluted sites in this study ranged from 0.003 to 3.686 μg/g. The higher values are similar to the Cd concentration of 3.6 $\pm$ 2.2 μg/g found in the liver of the bat *Eptesicus diminutus* (family Vespertilionidae) caught at metal-polluted coal mining areas of Brazil (Zocche *et al.* 2010). These Brazilian bats also showed significant metal-induced DNA damage (Zocche *et al.* 2010). On the other hand, Cd has a tendency to accumulate in tissue and Cd concentration is therefore strongly correlated with exposure time (Fritsch *et al.* 2010). Furthermore, Cd concentrations in the kidney and liver of small mammal species increase with age (Walker *et al.* 2007; Fritsch *et al.* 2010). Thus, given enough time, Cd levels in *N. nana* foraging at wastewater-polluted sites may reach critical concentrations. Because in our study, the Cd recovery from *N. nana* tissue was poor, the Cd concentrations given here may be an underestimation of the true extent of the accumulation.

Cr and Ni levels were below detection in the liver of *N. nana*, but concentrations in the kidney and muscle tissue ranged from 0.007–0.683 μg/g and 0.015–19.656 μg/g, respectively. Cr has been linked to chromosomal aberrancy (at mean concentrations ~3.053 μg/g) (Tull-Singleton *et al.* 1994) and carcinogenicity (O'Brien *et al.* 2003). In mammals, chronic exposure to Ni may cause degenerative effects in various organs (Sheffield *et al.* 2001). Although terminal physiological damage is not likely to occur from the Cr and Ni concentrations found in *N. nana*, there remains a risk of undetected sublethal effects. In fact, the upper limits of Cr and Ni concentrations in the kidney were higher than what was reported in meadow voles (*Microtus pennsylvanicus*) collected at a site treated with municipal sewage sludge (Alberici *et al.* 1989). What may be of significance here is that both Ni and Cr are readily transferred from adult to young through lactation (Streit & Nagel 1993).

The low abundance of *N. nana* at unpolluted upstream sites is also reflected in our relatively low capture rate here. Hence, sample sizes of metal concentration and diet of *N. nana* were relatively small at upstream sites. Contaminant concentration data obtained from tissue samples often contain a large degree of natural variability between individuals due to genetic variation and physiological fluctuations (Rothery 2000). Also, there can be much variability in the diet of bat species within the same habitat due to seasonal variation in the spatial and temporal availability of prey (Jacobs *et al.* 2007; Schoeman & Jacobs 2011). Thus, with increased sample size there may be a continuum of variation and increased overlap in diet and metals in tissues between wastewater-polluted and unpolluted sites.

Future research should also use methods with a lower detection limit for metal determination than ICP-OES, such as ICP-MS or differential pulse anodic stripping voltammetry (Pikula *et al.* 2010) to obtain a better resolution of accumulated metals in tissues. Although only adults were included in the metal analysis, sampled adult bats were representative of the population and may therefore have contained older bats. The correlation between age and the concentration of some metals such as Cd thus warrant a precise determination of age in future studies. In addition, metal concentrations should also be tested in insects captured at the polluted and unpolluted sites to more accurately track the transfer of metals from the pollutant source to the predator.

In conclusion, our study is the first to find evidence both for the small African insectivorous bat, *N. nana*, exploiting high abundance of pollutant-tolerant insects at WWTWs, and metal contamination in tissues of these bats. These urban adapters benefit in the short-term by exploiting the high abundance of metal-tolerant chironomid midges occurring at metal-polluted WWTW sites. However, there may also be long-term costs. Based on metal concentrations in the water and target organs, it can be concluded that metals from

wastewater are probably passing from the water through the food chain to *N. nana*. More specifically, important toxic metals, such as Cd, Cr and Ni, may accumulate in organs, pertinently the kidneys, potentially posing negative long-term health effects for both adult and young *N. nana*. Further research investigating specific physiological effects, such as lesions from metal exposure in the kidney and liver, and consequent health effects is currently being conducted in our laboratory.

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Chapter 3

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

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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

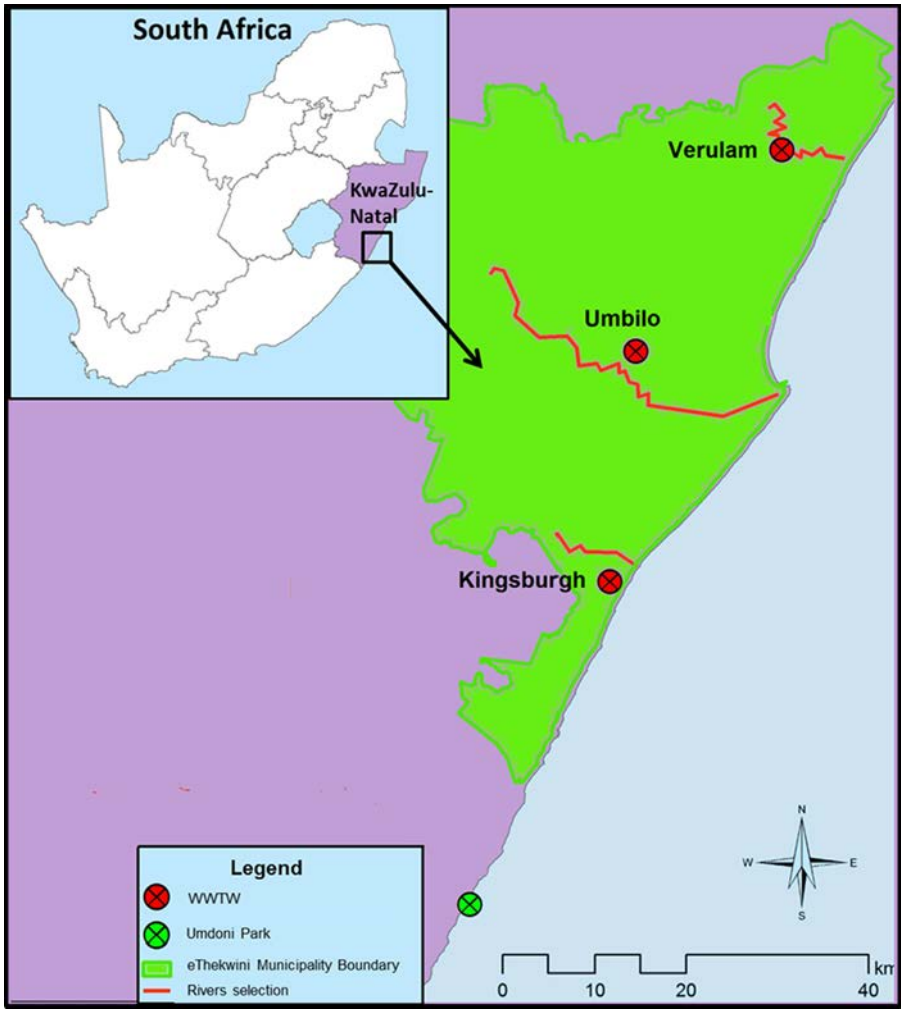

Fig. 1. Map of the study area in Durban, South Africa, showing the location of the Verulam, Kingsburgh and Umbilo Wastewater Works and Umdoni Park (unpolluted sites 1 and 2).

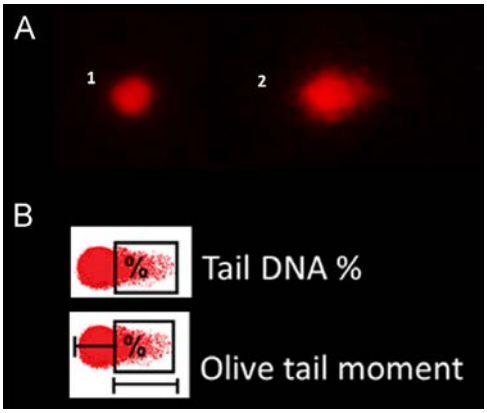

Fig. 2. (A) (1) cell containing undamaged DNA and (2) cell containing damaged DNA, visualised as a comet head and tail. (B) Visual representation of comet parameters (Tail DNA percent and Olive tail moment) measured.

protein assay kit. We added 20 μL of protein per bat and 20 μL of a series of $FeSO_2.7H_2O$ standards (0, 25, 50, 75, 100, 150, 200, 500, 1000 μM) in duplicate into a 96 well microtiter plate. 150 μL of FRAP working reagent (acetate buffer: TPTZ: $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio) was added to each well. We mixed reagents on a plate shaker and incubated for 20 min (Berker et al., 2007) at 37 °C, before allowing to cool. We confirmed that 20 min incubation allowed adequate time for the reaction to reach a steady-state. Absorbances were measured at 600 nm on a BioTek PowerWave XS multiwell plate reader and compared with a standard curve of Fe^{II} to determine the FRAP value for total antioxidant capacity.

2.4. Micronucleus assay

During erythrocyte maturation in mammals, the nucleus is expelled, resulting in an anucleated mature erythrocyte. However, remnants of nuclear chromatin resulting from chromosome breakage and/or aberrant cell division can still be observed as micronuclei in the otherwise anucleated erythrocytes (Hartmann et al., 2008).

We prepared peripheral blood smears on glass microscope slides using 10 μL of the blood/EDTA solution per slide. The slide was then air-dried, heat-fixed and stained with May Grunwald–Giemsa stain. We prepared two slides per individual. Criteria following Schmid, 1975, were used to identify and count micronuclei. We scored the frequency of micronucleated erythrocytes (MNE) in 2000 peripheral erythrocytes per individual using a Nikon Eclipse E400 microscope (magnification = 1000 ×, oil immersion). The peripheral erythrocytes scored included normochromatic (mature) and polychromatic (immature) erythrocytes and the assay was performed according to the EPA: U.S. Environmental Protection Agency (1998) guidelines specific for mammals. Parasitic infections such as malaria could resemble MN, but Dertinger et al. (2000) indicated that parasites are very prevalent in circulation, whereas whereas true MN are extremely rare. All animals exhibiting extremely highly prevalent MN-like material were excluded from the analysis.

2.5. Haematocrit

Whole peripheral blood was collected in heparinized micro-capillary tubes and stored on ice. The samples were centrifuged in a Heraeus Christ combifuge for 5 min at 6798 × *g*. The volume fraction of erythrocytes was measured on a Heraeus Christ micro-haematocrit reader.

2.6. Statistical analyses

We performed One-way ANOVAs and Tukey HSD post-hoc tests to compare differences in *N. nana* between WWTW sites and the unpolluted sites for OTM,

number of cells per damage class, TAC, number of MNE and percent haematocrit. In addition, we repeated the above ANOVAs on the standard deviations of the data to assess differences in interindividual variation associated with polluted sites (Depledge and Lundebye, 1996). We conducted simple linear regressions to determine the extent to which OTM at the different sites were attributed to percent tail DNA and tested whether there was a significant difference between the slopes using a One-way ANOVA. The OTM was compared with the percent haematocrit using a Pearson correlation. We also conducted a Spearman correlation between OTM and TAC to investigate whether there was a relationship between DNA damage and antioxidant capacity. Assumptions of normality and equality of variance were tested using a 1-sample Kolmogorov–Smirnov Test and a Levene's Test, respectively. Non-parametric tests were performed where assumptions were not satisfied. All analyses were performed with SPSS 21.0, using alpha of 0.05.

3. Results

3.1. DNA damage

The mean OTM/bat was significantly different between sites ($F_{(4,19)}=3.018$, $P=0.044$) with the Umbilo WWTW having a significantly higher OTM than unpolluted site 2 (Tukey HSD post-hoc test: $P=0.024$; Fig. 3a). Mean OTM/bat at the Umbilo WWTW remained significantly higher than the unpolluted sites combined (Tukey HSD post-hoc test: $P=0.024$). In addition, OTM was significantly correlated with percent haematocrit ($P=0.006$, $r^2=0.542$). The slopes of the simple linear regressions performed for OTM plotted against percent tail DNA were not significantly different between sites. However, the order of increasing magnitude of slopes and thus higher occurrence of double stranded DNA breaks were: unpolluted site 2 < unpolluted site 1 < Kingsburgh WWTW < Verulam WWTW < Umbilo WWTW (Fig. 3b). The average standard deviation of the OTM was higher at the Umbilo WWTW (mean OTM std. dev.=1.25) than at the unpolluted sites (mean OTM std. dev.=0.75), albeit not statistically significant ($P>0.05$). There was no significant difference between the sites for each damage category. However, DNA damage in WWTW bats was significantly higher than at the combined unpolluted sites for class 4 (40–95 percent) (Fig. 3c).

3.2. Total antioxidant capacity (TAC)

Total antioxidant capacity represented by the FRAP value was significantly higher at unpolluted site 1 than at all WWTWs ($F_{(4,38)}=3.005$, $P=0.033$; Tukey HSD post-hoc tests: $P<0.05$; Fig. 4). When the unpolluted sites were combined, the TAC was significantly higher than at all three WWTWs ($F_{(3,38)}=4.123$, $P=0.014$). There was no significant correlation between TAC and OTM ($P=0.797$, $r^2=0.066$), although in general the animals at WWTWs had lower TAC and higher OTM than unpolluted sites.

3.3. Micronucleus assay

The incidence of micronucleated erythrocytes for bats from each of the sites are summarized in Table 1. MNE were infrequent among individuals, with a mean occurrence of 0.16 MNE/1000 erythrocytes at all sites, except for the Umbilo and Verulam WWTWs which had mean of 0.25 MNE/1000 erythrocytes. Because no micronuclei were encountered for numerous individuals, statistical analysis could not be performed.

3.4. Haematocrit

There was a significant difference in mean haematocrit between sites ($F_{(4,44)}=9.614$, $P<0.001$). The mean haematocrit per bat was significantly higher at all three WWTWs than at unpolluted site 1, and at the Umbilo and Kingsburgh WWTWs than at unpolluted site 2 (Tukey HSD post-hoc tests: all $P<0.05$; Fig. 5). When unpolluted sites 1 and 2 were pooled, there was still a significantly higher mean haematocrit between sites ($F_{(3,49)}=9.508$, $P<0.001$). However, Tukey HSD post-hoc tests were

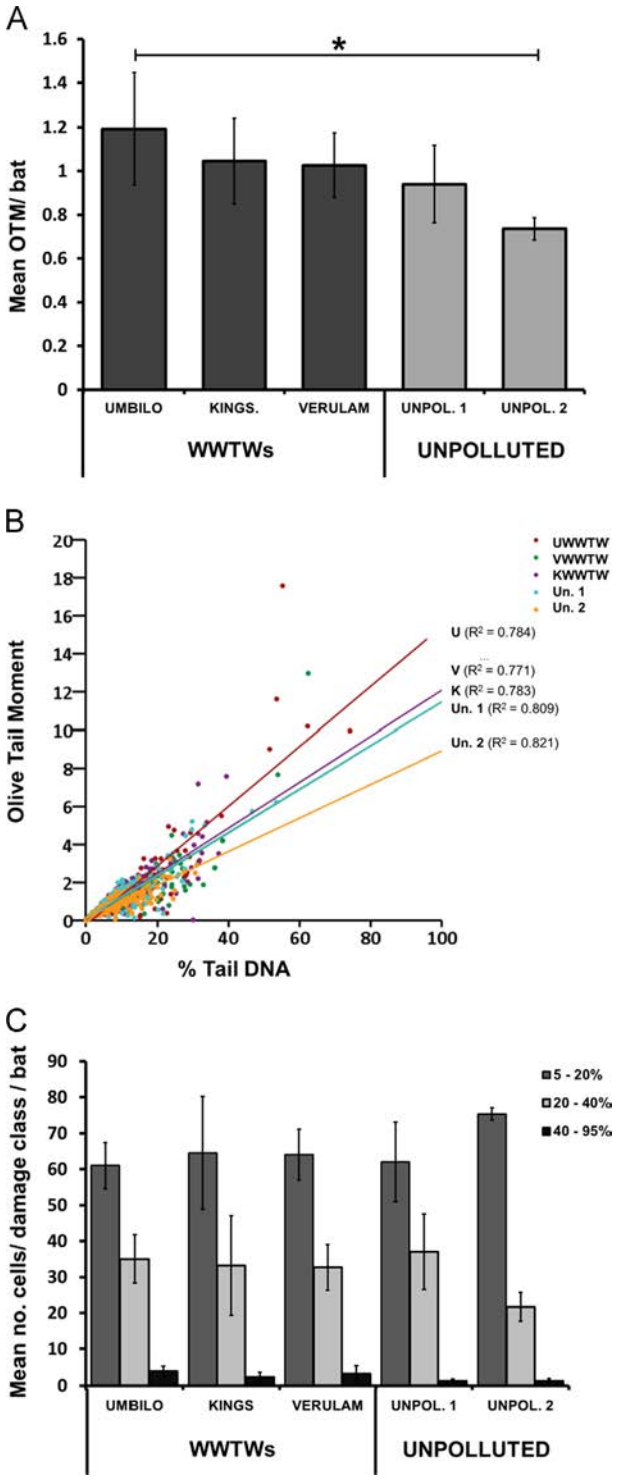

Fig. 3. (A) DNA damage, indicated by the mean Olive tail moment/bat, (B) relationship between Olive tail moment and percent Tail DNA (U=UWWTW=Umbilo WWTW; V=VWWTW=Verulam WWTW; K=KWWTW=Kingsburgh WWTW; Unpol. 1=Unpolluted site 1; Unpol. 2=Unpolluted site 2) and (C) Mean number of cells per DNA damage class per bat in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park (* indicates significant differences between the sites at the $P<0.05$ level). $N=24$; Bars= ± Std.dev.

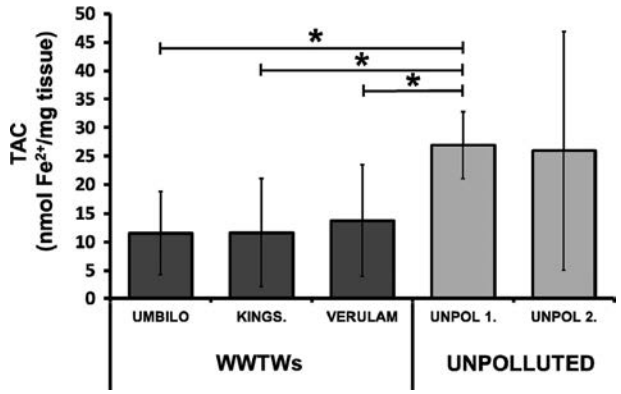

Fig. 4. Total antioxidant capacity in *N. nana* pectoral muscle tissue at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 (Unpol. 1 and Unpol. 2) at Umdoni Park (* indicates significant differences between the sites at the $P < 0.05$ level). $N = 37$; Bars = ± Std.dev.

Table 1
Number of micronucleated peripheral erythrocytes (MNE) in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park.

Site	No. of bats	MNE/bat	Mean MNE/bat	MNE/1000 erythrocytes
Umbilo WWTW	6	0/2/0/0/1/0	0.5	0.25
Kingsburgh WWTW	6	0/1/0/0/1/0	0.3	0.16
Verulam WWTW	6	1/0/1/0/0/1	0.5	0.25
Unpolluted site 1	3	0/0/1	0.3	0.16
Unpolluted site 2	3	0/1/0	0.3	0.16

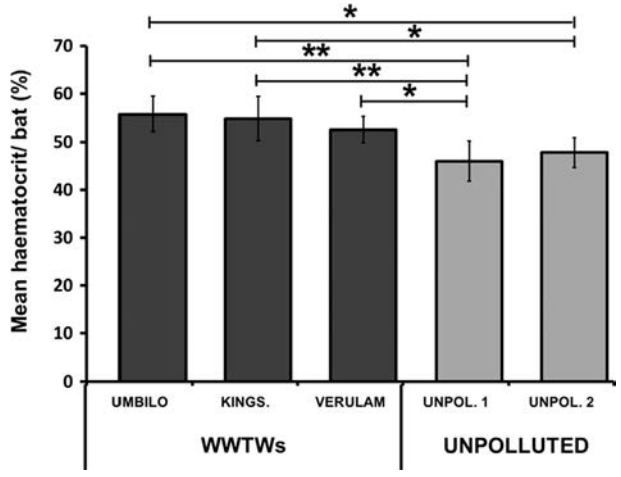

Fig. 5. Mean haematocrit per bat in *N. nana* at the Umbilo, Kingsburgh (Kings.), Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park (Unpol. 1 and Unpol. 2) (* and ** indicate significant differences between the sites at the $P < 0.05$ and $P < 0.001$ levels, respectively). $N = 48$; Bars = ± Std.dev.

significantly higher at only the Umbilo WWTWs than at the unpolluted site ($P < 0.001$).

4. Discussion

We predicted that pollutant exposure from foraging at WWTWs would elicit haematological and genotoxic responses in *N. nana*. In accordance with our predictions, we found significantly higher DNA damage, and diminished total antioxidant capacity in bats foraging at WWTWs, and haematocrits were also significantly higher in these bats. DNA damage from pollutants, namely metals such as those found in wastewater, occurs primarily by oxidation of metals into metal ions that have a high affinity for amino acids and which in addition, results in the generation of damaging free radicals or ROS. DNA damage may also occur from interference of DNA repair processes caused by pollutants (Hartwig, 1995). Bats however, produce comparatively lower ROS and higher concentrations of antioxidants to compensate for the excess ROS produced during flight (Salmon et al., 2009) and to minimize oxidative stress when transitioning from torpor to an active state (Wilhelm Filho et al., 2007). For example, both Mexican free-tailed bats (*T. brasiliensis*) and Cave Myotis bats (*Myotis velifer*) showed higher tolerance to protein oxidation compared to mice (Salmon et al., 2009). In addition, *Myotis daubentonii* exhibited tolerance to oxidative damage from an organic tin compound, tributyltin (Lilley et al., 2013). In fact, the longevity of bats which is three times longer than mammals of a similar basal metabolic rate, is largely facilitated by this lower production of free radicals together with their increased antioxidant defences (Brunet-Rossinni and Austad, 2004; Brunet-Rossinni, 2004a, 2004b; Wilhelm Filho et al., 2007). However, low levels of polyunsaturated fatty acids in the midges (Ghioni et al., 1996) that the bats feed on at WWTWs (Naidoo et al., 2013), may not provide adequate protection against formation of ROS during arousal from torpor.

The slope of the regression of OTM and percent Tail DNA indicated that the nature of the DNA damage in WWTW bats consisted of more double stranded than single stranded breaks. In addition, DNA damage in WWTW bats was significantly higher within class 4 (40–95 percent), further indicating a high proportion of severe damage such as double stranded DNA breaks. Double stranded DNA breaks are of more concern than single stranded breaks because they can affect multiple genes simultaneously, may disrupt cell-cycle regulation, lead to cell malfunctioning, and ultimately, cause cell death (van Gent et al., 2001). In addition, double stranded breaks require higher DNA repair efforts (Hartwig, 1998).

Metal ions in particular, not only generate DNA damage with high repair requirements, but also inhibit the proteins involved in DNA repair itself (Hartwig, 1998; Hartwig and Schwerdtle, 2002). Some metal ions are more damaging to DNA and DNA repair mechanisms than others (Hartwig, 1998). Of the toxic metals commonly found in WWTWs, cadmium, nickel and lead, even at low concentrations, inhibit DNA repair. Chromium and chromium compounds, especially hexavalent chromium, are particularly damaging to DNA due to its unique action of direct binding to DNA in addition to ROS production (Beyersmann and Hartwig, 2008). This has been observed in chromium exposed humans working in the chrome-plating industry who had elevated DNA strand breaks compared to non-exposed workers (Gambelunghe et al., 2003). Notably, DNA damage and chromium concentrations were significantly higher at Umbilo than at the other two WWTWs (Naidoo et al., 2013). Furthermore, metals (Cd, Ni, Cr, Fe, Cu, Zn) quantified in the kidney of bats foraging at these WWTWs correlated to metal levels in the water at the sites, with kidney Cd at the Umbilo WWTW measured at concentrations as high as 3.686 $\mu g\, g^{-1}$—similar to levels (4.05 $\mu g\, g^{-1}$) found in Brazilian bats with significant DNA damage (Zocche et al., 2010). Although not statistically significantly different, the average standard deviation for OTM/individual was also higher at the Umbilo WWTW than at the unpolluted sites. This greater inter-individual variability is characteristic of parameters measured in individuals from contaminated sites (Depledge and Lundebye, 1996). In fact, all negative impacts assessed were most apparent at the Umbilo WWTW, which has one of the lowest scores for wastewater quality compliance in KwaZulu-Natal (DWAF: Department of Water Affairs and Forestry, 2009). Measuring total DNA strand breaks cannot however, provide insight into the specific cause or mechanism of damage, and the higher DNA damage at WWTWs

could have been caused or exacerbated by nutritional status (Ames, 1999), disease and age (for review see Fairburn et al., 1995), none of which was quantified in detail here.

Bats foraging at the Umbilo WWTW showed the lowest antioxidant capacity where TAC was significantly lower than at the unpolluted sites. Antioxidant systems such as superoxide dismutase, catalase, and glutathione peroxidase are vital in counteracting damage from the production of ROS, by scavenging free radicals. Even though bats already have high baseline antioxidant enzymes (Wilhelm Filho et al., 2007), they can further enhance the activity of these enzymes to provide protection against harmful ROS (Arenas-Ríos et al., 2007). However, wastewater-associated metals have the ability to impair antioxidant activity (Beyersmann and Hartwig, 2008). For instance, cadmium inhibits catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase (Beyersmann and Hartwig, 2008). The TAC is a simple measure of total reducing power or plasma or tissue extracts, without providing any information on the levels of each component of the antioxidant defence system (Halliwell and Gutteridge, 2007). TAC, as measured with the FRAP assay, therefore does not represent all aspects of redox regulation (Prior and Cao, 2001). The significantly lower TAC at WWTWs, suggests that foraging at these sites, at least in part, influences the maintenance of redox regulation in the bats, and a more detailed analysis of redox state may be warranted.

There was no correlation between OTM and TAC. OTM was however, measured in blood, whereas TAC was measured in muscle. These parameters, in addition to other factors such as DNA repair rate should be measured within the same tissue type to investigate the relationship between DNA damage and antioxidant capacity. The fact that DNA integrity is diminished in at least one tissue type of bats foraging at sites where TAC is also significantly lower than that of reference sites, is in itself worrying.

Bats at WWTWs had significantly higher haematocrits than bats at unpolluted sites. A higher haematocrit may be attributed to an increased erythrocyte turnover in response to a variety of factors including nutritional stress, hydration and parasitism (Decker and Knight, 1990). For instance, high production rate of erythrocytes has been noted in bats as a stress response to experimentally imposed social isolation combined with cold exposure (Martin and Stehn, 1977). Whether haematocrits increase or decrease with exposure very much depends on the stressor. For instance, wood mice, *Apodemus sylvaticus* exposed to soil metal pollution had lower haematocrits than unexposed mice (Rogival et al., 2006). Conversely, Cyriac et al. (1989) found *Oreochromis mossambicus* fish to have an increased haematocrit in response to metal exposure and attributed it to swelling of erythrocytes. Different metals may however, affect erythrocyte production differently. For instance, lead (Bersényi et al., 2003) and cadmium (Zikic et al., 2001) exposure result in lower haematocrit, whereas an excess of iron may cause an increase in haematocrit (Crowe and Morgan, 1997). Our previous study (Naidoo et al., 2013) found iron levels at the tank sites of both the Umbilo (0.9 ± 0.07 mg L^{-1}) and Verulam (1.3 ± 0.00 mg L^{-1}) WWTWs to be significantly higher than at downstream sites. To put this into perspective, both concentrations were higher than the South African Water Quality Guideline (DWAF: Department of Water Affairs and Forestry, 1996) for human consumption (0–0.1 mg L^{-1}), and it may therefore be quite plausible for bats foraging at these sites to have high haematocrit values, typical of tissue iron overload. Iron-catalysed oxidative stress can also result in DNA damage (Johnson, 2000), thereby at least in part explaining the significant correlation between OTM and haematocrits in *N. nana*.

We found no increase in micronucleus formation in erythrocytes of bats at WWTWs, and thus no observed chromosomal aberration. This indicates that cellular functioning has not been disrupted by pollutant exposure. Pollutants may cause the production of lagging/centromere-lacking chromosomic fragments or may affect the functioning of the spindle apparatus (Schmid, 1975). Micronuclei are formed after the telophase stage of cell division when the lagging chromosomic fragments remain in the cytoplasm of daughter cells as a small, secondary nucleus in most vertebrates and a small piece of nuclear material in the otherwise anucleated erythrocytes of mammals (Hartmann et al., 2008). The normal resting values for the frequency of micronucleated erythrocytes differs widely among species (eg. Lynx (*Lynx ruffus*)= 1.08; Crocodile (*Crocodylus moreletty*)=0.3; Squirrel (*Spermophilus variegatus*)=0.05 MNE/1000 erythrocytes) (Zúñiga-González et al., 2000, 2001). The MNE values obtained for *N. nana* (0.16–0.25 MNE/1000 erythrocytes) were similar across all sites and are thus most likely representative of the normal, baseline range of MNE frequency for this species. Similarly, spontaneous MNE for the Jamaican fruit bat, *Artibeus jamaicensis* were 0.1 MNE/1000 erythrocytes (Zúñiga-González et al., 2000). The constant, low level of MNE in *N. nana* may indicate that bats have an efficient MNE removal system, and/or the spleen of *N. nana* at WWTWs removed defective erythrocytes such as MNE from the circulating blood at an increased rate, replacing them due to the high turnover of new erythrocyte production (Corazza et al., 1990).

To conclude, the increased haematocrit and DNA damage in peripheral blood, in conjunction with diminished antioxidant capacity in muscle tissue suggest that foraging at WWTWs affects multiple levels of physiology causing potentially harmful responses in *N. nana*. DNA damage, especially double stranded breaks, may result in an accumulation of mutations which ultimately lead to tumour formation, cancers and other hereditary diseases (Pastink et al., 2001; van Gent et al., 2001). Thus, short-term benefits from abundant prey carry long-term risks through the occurrence of trans-generational epigenetic changes (Martinez-Zamudio and Ha, 2011). This together with our previous study (Naidoo et al., 2013) 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. This remains an important focus for further studies in our labs.

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Poster title:

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

LIVE FAST, DIE YOUNG:
Haematological and genotoxic responses in an urban adapter, the Banana Bat, foraging at wastewater treatment works

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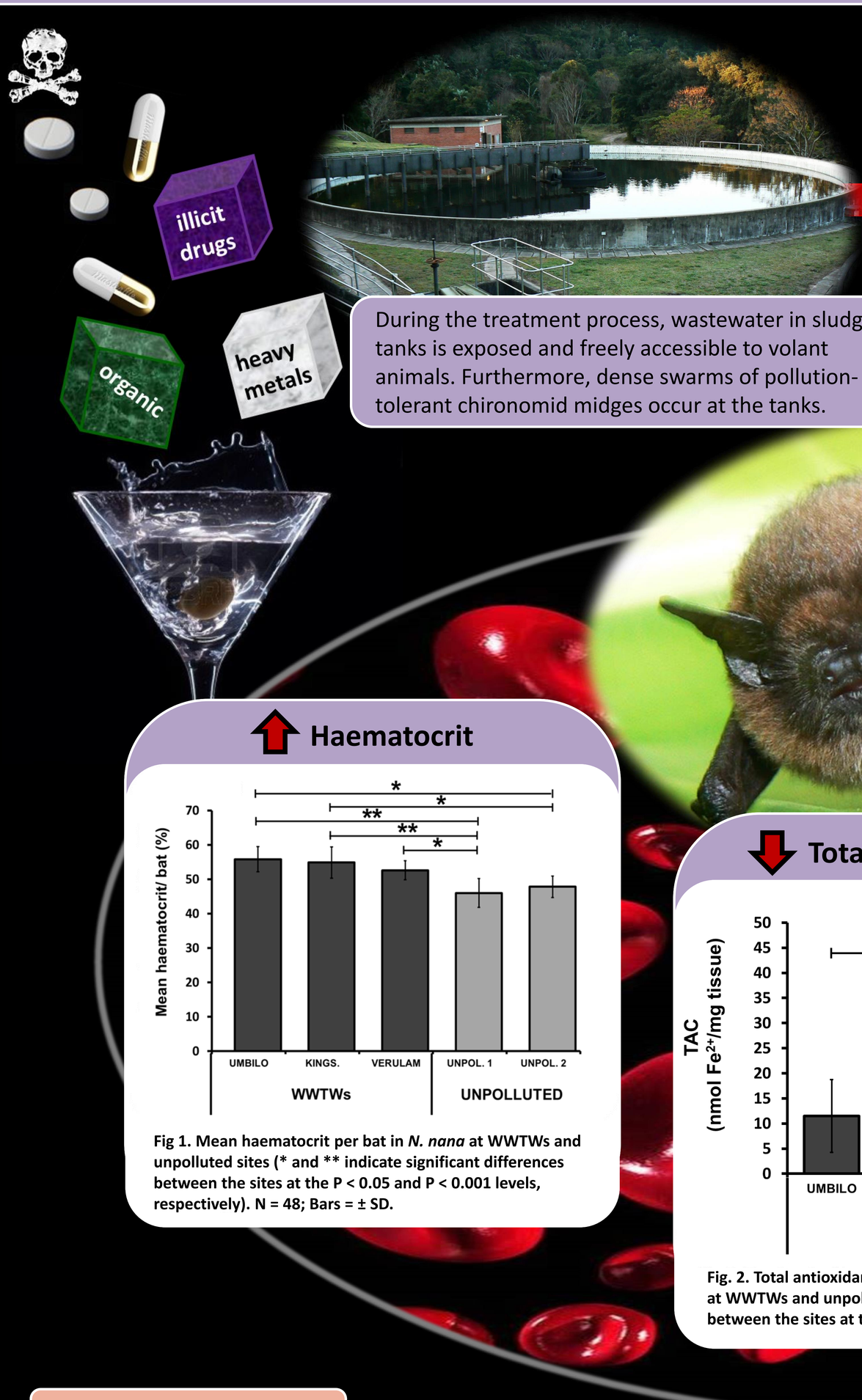
Background

To cater for the rapidly growing human population, natural land is being transformed into urban habitat at an alarming rate [1]. Wastewater Treatment Works (WWTWs) are a ubiquitous feature of the urban landscape. They receive a cocktail of pathogens, inorganic and organic contaminants which undergo various stages of treatment in large, open-top sludge tanks before being discharged into rivers [2]. The Banana Bat, *Neoromicia nana* is an urban adapter that specifically exploits insect prey at polluted sites [3], and is the most abundant bat species at WWTWs in KwaZulu-Natal, South Africa [4]. We investigated whether pollutant exposure from foraging at WWTWs elicits haematological and genotoxic responses in *N. nana*. We compared four measures of haematological/ genotoxic damage between *N. nana* foraging at three WWTWs (Umbilo, Kingsburgh, Verulam WWTWs) and two unpolluted sites (within the Umdoni Park) located in KwaZulu-Natal: blood oxygen capacity based on haematocrits, chromosomal aberration indicated by micronuclei formation, total antioxidant capacity as indicated by the FRAP assay and DNA damage measured by the comet assay.



During the treatment process, wastewater in sludge tanks is exposed and freely accessible to volant animals. Furthermore, dense swarms of pollution-tolerant chironomid midges occur at the tanks.

N. nana exploits the high abundance of chironomid midge prey available at WWTWs, with Chironomidae comprising the highest proportion of their diet at tanks [4]. Chironomid midges, however, can accumulate high levels of metals, without decreases in survival & growth to the adult stage.

N. nana at WWTWs thus have higher levels of non-essential metals in their tissues than bats at unpolluted sites. In addition, metal levels in the kidneys correlate to metal levels at the polluted sites [4].

Haematocrit

Fig 1. Mean haematocrit per bat in *N. nana* at WWTWs and unpolluted sites (* and ** indicate significant differences between the sites at the P < 0.05 and P < 0.001 levels, respectively). N = 48; Bars = ± SD.

Total antioxidant capacity

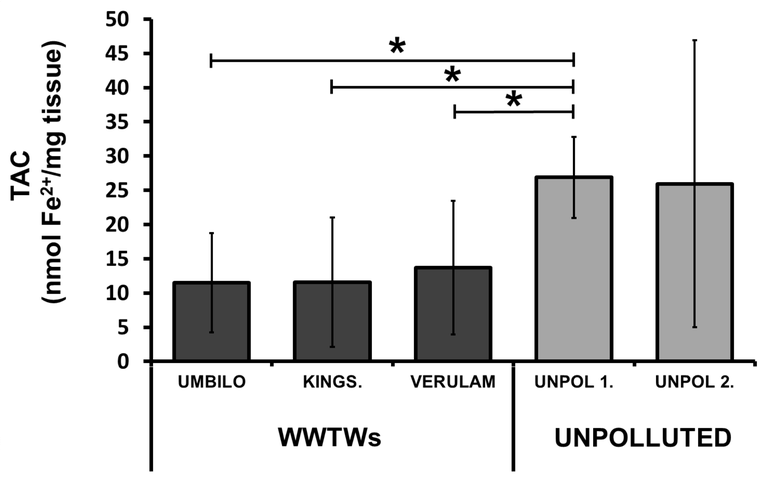
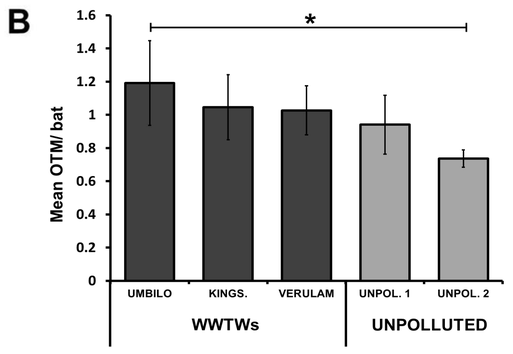


Fig. 2. Total antioxidant capacity in *N. nana* pectoral muscle tissue at WWTWs and unpolluted sites (* indicates significant differences between the sites at the P < 0.05 level). *N* = 37; Bars = ± SD.

DNA damage



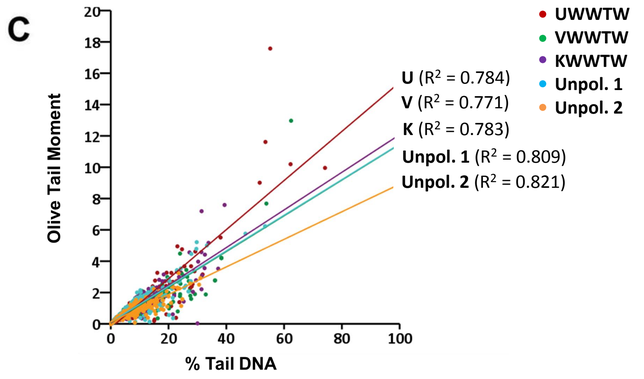


Fig 3. (A) Healthy/ damaged DNA in cell, visualised as a comet head and tail, and representation of Olive tail moment (OTM), (B) DNA damage, indicated by mean Olive tail moment/ bat, (C) relationship between OTM and % Tail DNA at WWTWs and unpolluted sites (* indicates significant differences between sites at P < 0.05 level). *N* = 24; Bars = ± SD.

Implications

WWTWs provide the short-term benefit of highly abundant prey for bats, however we found evidence that pollutant exposure from foraging at WWTWs elicits haematological and genotoxic responses in *N. nana* [5]. The increased haematocrits in *N. nana* at WWTWs (Fig. 1), may be due to erythrocyte production in response to certain pollutants. There was no increase in micronucleus frequency in WWTW bats, indicating that cellular functioning has not yet been disrupted by chemical exposure. However, WWTW bats had a significantly lower antioxidant capacity than bats from unpolluted sites (Fig. 2). 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. In addition, the higher DNA damage in *N. nana* at WWTWs than in bats from unpolluted sites (Fig. 3B), and the slope of the regression of OTM and %Tail DNA (Fig. 3C) suggests inadequate repair to double stranded DNA breaks. These results suggest that foraging at WWTWs affects multiple levels of physiology causing potentially harmful responses such as tumour formation, cancers and other hereditary diseases [6]. Thus, WWTWs, that are aimed to remove pollutants from the environment, can themselves act as a source of contamination and pose serious long-term threats to animals exploiting these habitats. This remains an important focus for further studies in our labs.

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Chapter 4

Pollutant exposure at wastewater treatment works affects the detoxification organs of an urban adapter, the banana bat

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Pollutant exposure at wastewater treatment works affects the detoxification organs of an urban adapter, the Banana Bat

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ABSTRACT

The Banana Bat, *Neoromicia nana*, exploits pollution-tolerant chironomids at wastewater treatment works (WWTWs). We investigated how pollutant exposure impacts the detoxification organs, namely the liver and kidney of *N. nana*. (i) We performed SEM-EDS to quantify metal content and mineral nutrients, and found significant differences in essential metal (Fe and Zn) content in the liver, and significant differences in Cu and one mineral nutrient (K) in the kidneys. (ii) We performed histological analysis and found more histopathological lesions in detoxification organs of WWTW bats. (iii) We calculated hepatosomatic/renalsomatic indices (HSI/RSI) to investigate whole organ effects, and found significant increases in organ size at WWTWs. (iv) We quantified metallothionein 1E (MT1E), using Western Blot immunodetection. Contrary to predictions, we found no significant upregulation of MT1E in bats at WWTWs. Ultimately, *N. nana* exploiting WWTWs may suffer chronic health problems from sublethal damage to organs responsible for detoxifying pollutants.

1. Introduction

The global human population has been growing at an unprecedented rate and is projected to reach over 9 billion by the year 2050 (United Nations Population Fund, State of World Population (2014)). To accommodate the growing numbers, natural land is rapidly becoming urbanized. In fact, in India, China and Africa, urban land expansion rates have now exceeded or are equal to urban population growth rates (Seto et al., 2011). The typical infrastructure of cities is designed to cater for the needs of the urban population, with the most basic requirement being sanitation. Thus, a common physical feature of the urban landscape is wastewater treatment works (WWTWs). WWTWs receive industrial and household waste which is treated in large, open-top tanks (Gagnon and Saulnier, 2003). Wastewater treatment tanks usually contain particularly high levels of metals (Karvelas et al., 2003). In the UK, an estimated 12,508 tonnes of the toxic elements Cd, Cu, Cr, Ni, Pb and Zn is received at a typical urban WWTW per year (Crane et al., 2010). Despite advances in metal treatment techniques, the removal of certain metals such as copper and zinc at WWTWs, have shown little improvement in the last three decades (Crane et al., 2010). A prominent biotic characteristic of WWTWs is the proliferation of pollution-tolerant chironomid midge swarms (Boonstra et al., 2009). Chironomid midges often contain high concentrations of metal pollutants from WWTWs without showing adverse effects on survival (Krantzberg and Stokes, 1990). Predators that feed on these midges may however, accumulate metals in their tissues with acute or chronic effects on their health (Hare, 1992).

The Banana Bat, *Neoromicia nana*, is an urban adapter (Jung and Kalko, 2011; Monadjem et al., 2010) that exploits the swarms of pollution-tolerant chironomid midges that occur at WWTWs (Naidoo et al., 2013). At wastewater-polluted sites, we previously found that chironomid midges were the most abundant prey type in the diet of resident bats (Naidoo et al., 2013), compared to diverse insect diets at unpolluted sites (Naidoo et al., 2013; Schoeman and Jacobs, 2011). Significant correlations between insects captured and diet composition at the wastewater-polluted sites suggested that *N. nana* fed opportunistically on the abundant chironomid prey (Naidoo et al., 2013). There is a considerable body of literature showing the transfer of metals and other pollutants from chironomid prey to predators, including bats (Goodyear and McNeill, 1999; Lescord et al., 2015; Park et al., 2009; Reinhold et al., 1999; Timmermans et al., 1992). In addition, metal concentrations in the water at the WWTW sites were significantly correlated with the metal concentrations in the kidney tissue of the bats (Naidoo et al., 2013). We have previously shown that pollutant

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exposure from this abundant food resource carries physiological costs for *N. nana*, specifically sub-lethal haematological and genotoxic responses (Naidoo et al., 2015). *N. nana* at WWTWs had significantly lower antioxidant capacity and significantly higher levels of DNA damage and haematocrits than bats from unpolluted sites (Naidoo et al., 2015). An accumulation of DNA damage, especially from double-stranded breaks as observed in *N. nana* at WWTWs, ultimately leads to tissue aberrations and disease (Jackson and Bartek, 2009). These longer-term effects may occur in various tissue types and organs in the body, however the organs where sub-lethal effects of chronic pollutant exposure would be most evident are the liver and kidneys (Clark and Shore, 2001).

The liver and kidneys are the main organs responsible for detoxification in the body. The liver has a wide range of functions including detoxification of the blood by excretion in bile, phagocytosis and chemical transformation of toxic molecules (Fox, 1991). The kidneys regulate the extracellular fluid environment in the body and the concentrations of waste products that are filtered from the blood and returned into circulation (Fox, 1991). Thus, when bats ingest pollutants including the metals found at WWTWs, they are either metabolized, excreted, accumulated or stored in a less toxic form (Baker et al., 2003). An accumulation of metals in the organs may, however, cause various types of tissue damage including inflammation, necrosis, hyperplasia or hypertrophy. These lesions in the tissue may further lead to altered organ size and impaired organ function (Ma, 1989).

Metallothionein 1 E (MT1E) is a protein produced primarily in the liver and kidney that protects against metal damage by binding to and detoxifying metal ions (Sakulsak, 2012). Metallothionein has a high affinity for non-essential metals such as Cd and Hg and some essential metals such as Zn and Cu, with its metal binding affinity in the order: Cd > Pb > Cu > Hg > Zn > Ag > Ni > Co (Waalkes et al., 1984). When the metal ions exceed metallothionein binding capacity, they may cause physical damage such as histopathological alterations (Goyer et al., 1989) to tissue as observed in both the liver and kidney (Sánchez-Chardi et al., 2009) of shrews (*Crocidura russula*) inhabiting an abandoned pyrite mining site. Thus, metallothionein is generally upregulated in animals exposed to excess metal levels (Dai et al., 2013; Sakulsak, 2012).

Metallothionein protein expression is however, highly species-specific (Henry et al., 1994). For instance, humans have metallothionein concentrations per gram of liver up to 100 times the levels of that found in rat and mouse (Henry et al., 1994). To date, of the limited number of reports of metallothionein levels in bats, Pikula et al. (2010) found that species and foraging habitat influences metallothionein content. Bats foraging in aquatic habitats had higher levels of metallothionein than bats foraging in terrestrial or terrestrial/aquatic-habitats (Pikula et al., 2010). Habitat quality and diet play a significant role in eliciting physiological coping responses. Thus, given that WWTWs form an integral part of the urban landscape and an important prey base for urban adapters, it is important to understand the potential sub-lethal effects in organs of *N. nana* foraging at these sites. Furthermore, the effect of exposure to the cocktail of pollutants at WWTWs has not been elucidated in wild bats or laboratory based studies.

The aim of our study was to therefore investigate how pollutant exposure impacts the detoxification organs, namely the liver and kidney of *N. nana* foraging at WWTWs: (i) We performed SEM-EDS metal imaging to quantify the content of metals and mineral nutrients in liver and kidney tissue; (ii) We performed histological analysis to investigate the extent of tissue damage in the detoxification organs; (iii) We calculated hepatosomatic/renalsomatic indices (HSI/RSI) to investigate whole organ effects and (iv) We quantified metallothionein 1E (MT1E) in the liver and kidney, using Western Blot immunodetection. We predicted that, compared to *N. nana* foraging at unpolluted sites, *N. nana* foraging at WWTWs should have (i) higher levels of toxic non-essential metals, (ii) a greater extent of histopathological lesions in the liver and kidney tissue, (iii) higher hepatosomatic/renalsomatic indices (characteristic of organ swelling due to metal damage (Ma, 1989)), and (iv) upregulated metallothionein protein content in the liver and kidney.

2. Methods

2.1. Sample collection

We captured *N. nana* bats at sludge tanks in three WWTWs within 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). These WWTWs use open-top sludge tank systems that contain high levels of wastewater–associated metals (lead, cadmium, chromium, nickel, copper, zinc and iron; Naidoo et al., 2013). We captured *N. nana* bats at two unpolluted reference sites in the forest of Umdoni Park (S30°41.15′; E30°23.35′), 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 WWTWs 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 WWTWs was unlikely.

We used mist nets and harp traps to capture bats at the sites during the summer (January–March 2013). We recorded their sex,

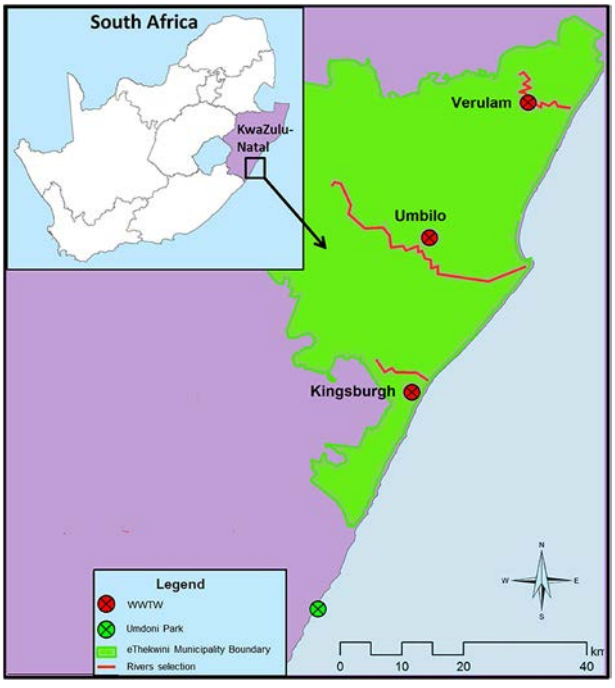

Fig. 1. Map of the study area in Durban, South Africa, showing the location of the Verulam, Kingsburgh and Umbilo Wastewater Works and Umdoni Park (Unpolluted sites 1 and 2).

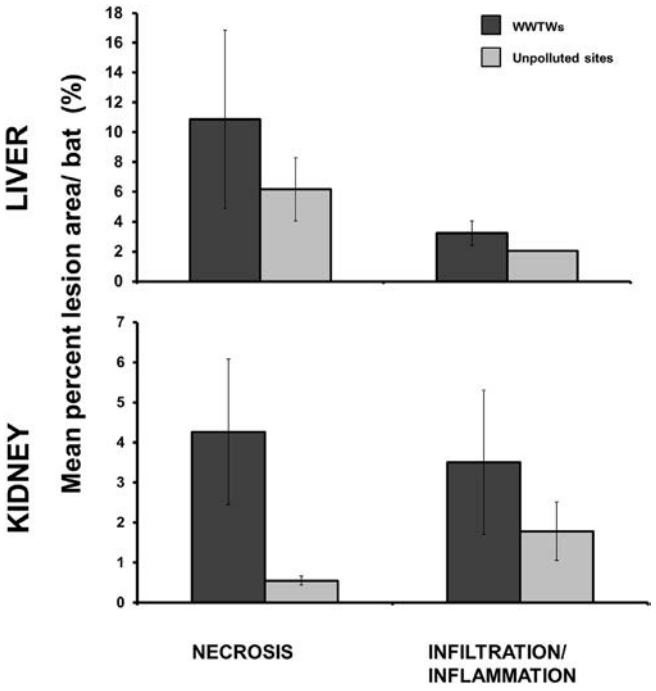

Fig. 2. Mean percent necrosis and infiltration/inflammation area in the liver and kidney tissue of *N. nana* at WWTWs and unpolluted sites (Bars = ±Std.dev).

forearm length (to nearest 0.1 mm), and body mass (to nearest 0.5 g). We assessed life-stage (juvenile or adult) from the presence of cartilaginous epiphyseal plates (Anthony, 1988). Species were identified using a taxonomic key (Monadjem et al., 2010) and only adult *N. nana* where kept for analyses. To minimize the number of animals sacrificed, we captured six bats per site as used in other physiological studies of bats (Pilosof et al., 2014). However, because *N. nana* is less abundant at unpolluted sites than at WWTWs (Naidoo et al., 2013, 2015), we collected three bats from each unpolluted site. The bats were humanely euthanized, as approved by the University of KwaZulu-Natal Animal Ethics Committee (Reference: 031/13/Animal).

2.2. Elemental content

Dissected liver tissue and right kidney tissue from each bat was stored on ice and then individually snap frozen by plunging into liquid nitrogen. The snap frozen samples were immediately stored at −80 °C. We dehydrated the tissue for three days in an Edwards Modulyo Freeze dryer (Edwards, United Kingdom). The samples were then fractured, and mounted onto specimen stubs with the fractured surface positioned upward. We coated the samples with a thin layer of carbon using a Quorum Q 150 TE carbon coater (Quorum Technologies, East Sussex, UK). We viewed and digitally captured images of the tissue with a Zeiss Field Emission Gun Scanning Electron Microscope Ultra Plus. Energy-Dispersive X-ray Spectrometric analysis (SEM-EDS) was performed using AZtecEnergy Version 1.2 (Oxford Instruments Analytical, Oxfordshire) software for the EDS detector (X-Max 80 mm, Oxford Instruments) connected to the electron microscope (operating at 20 kV).

SEM-EDS bombards the sample with electrons to generate X-rays. An energy spectrum of peaks, characteristic of the elements of interest, is quantified to produce the relative percentage elemental composition of the sample. We performed compositional analyses for the following metals: aluminium (Al), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb), and mineral nutrients: nitrogen (N),oxygen (O), sodium (Na), magnesium (Mg), phosphorous (P), potassium (K) and calcium (Ca). Three technical replicates per compositional analysis were run for each liver/kidney collected from individual bats, at a standardized working distance of 8.5 mm (magnification = 400×).

2.3. Histological evaluation

We fixed a piece of dissected liver and the left kidney from each bat in 10% neutral-buffered formalin until dehydration in an ethanol dilution series. The tissues were then cleared in xylene and embedded in paraffin wax. We prepared slides of hematoxylin and eosin stained, 5–10 mm sections of liver and kidney. We captured three images/slide using a Nikon Eclipse E400 microscope (magnification = 400×) connected to a Nikon E5400 camera. We scored liver and kidney tissue damage per individual based on total number of lesions, total number of lesion types and percent lesion area relative to tissue area. Lesions were scored using digital whole-slide imaging on Leica SlidePath Gateway 2.0 (Leica Microsystems, Germany).We took care to differentiate between lesions and autolysis associated with specimen fixation, which may mimic necrosis (Shackelford et al., 2002).

2.4. Hepatosomatic/renalsomatic index

The liver and both kidneys were dissected from each bat and kept moist to prevent dehydration. They were immediately weighed on a three decimal balance (wet weight - to nearest 0.001 g). Hepatosomatic and renalsomatic indices were calculated as a percentage of the organ weight relative to the total body weight of each individual (organ weight/total body weight × 100) (Sellers et al., 2007).

2.5. Metallothionein content

To quantify metallothionein 1E (MT1E) protein content, dissected liver tissue and right kidney tissue from each bat was stored on ice and then individually snap frozen by plunging into liquid nitrogen. The snap frozen samples were immediately stored at −80 °C until used for whole cell extraction. We added Halt™ protease inhibitor cocktail (Thermo Scientific) to each sample to prevent protein degradation. The tissue was weighed and homogenized using an extraction buffer as in Mosser et al. (1988). Tissue was disrupted in a TissueLyser (Qiagen, Germany) and centrifuged at 4 °C for 10min, at 8050× g. Protein concentrations in the supernatant acquired were quantified using a Pierce BCA protein assay kit (Thermo Scientific) and BioTek PowerWave XS multiwell plate reader and KC4 software (Bio Tek). We used the extracted protein to perform Western Blot immunodetection on all liver

Table 1
Total number of lesions (Total #), type of lesions (Lesion types: (a) necrosis, (b) infiltration/inflammation, (c) vacuolization, (d) hyperplasia, (e) atrophy, (f) tubular dilatation, and (g) cylinders), and percentage individuals showing lesions (% Indiv.) in the liver and kidney tissue of *N. nana* at the Umbilo (UWWTW), Kingsburgh (KWWTW), and Verulam (VWWTW) WWTWs and unpolluted sites 1 (UNPOL. 1) and 2 (UNPOL. 1) at Umdoni Park.

	LIVER lesions			KIDNEY lesions		
	Total #	Lesion types	% Indiv.	Total #	Lesion types	% Indiv.
UWWTW	5	a, c	83	5	a, b, f, g	67
KWWTW	4	a, b, c	67	4	a, b	67
VWWTW	10	a, b, d, e	67	9	a, b, g	100
UNPOL. 1	1	a	33	2	a, b	33
UNPOL. 2	2	a, b	67	1	a	33

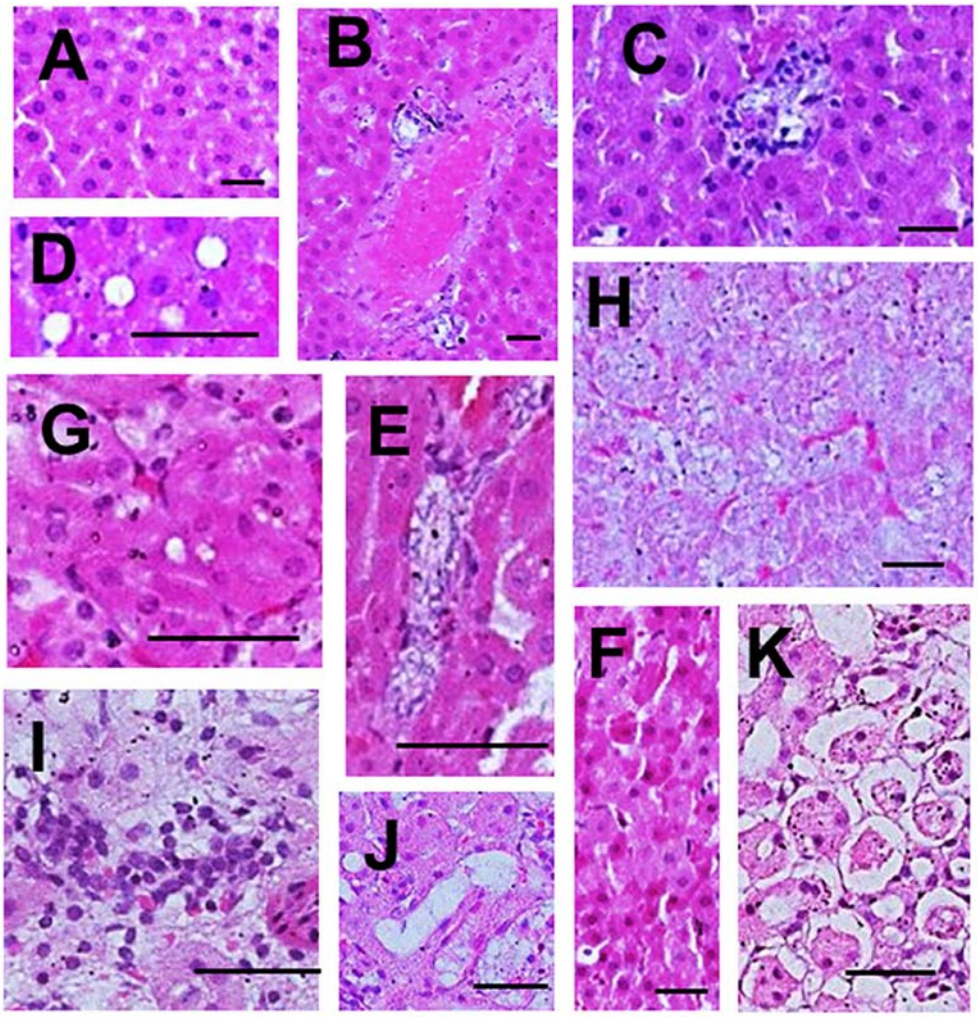

Fig. 3. *N. nana* hepatic (A–F) and renal (G–K) sections showing: (A, G) tissue without lesions, (B, H) necrosis, (C, I) infiltration/inflammation, (D) vacuolization, (E) hyperplasia, (F) atrophy, (J) tubular dilatation, and (K) cylinders (H&E, ×400; scale bars represent 50 μm).

samples and subsequently, on all kidney samples. 20 μg of extracted protein for each sample was diluted with sample loading buffer (buffer: sample in a 1:6 ratio). $CdCl_2$ was added to the sample solution at a concentration of 0.25 mg Cd/ml, to facilitate the migration of MT as a compact band (Aoki and Suzuki, 1991). The sample solution was boiled for five minutes at 95 °C and briefly cooled on ice. Proteins and a PageRuler pre-stained protein ladder (Thermo Scientific) were separated on an 8% sodium dodecyl sulphate polyacrylamide gel at a voltage of 90 V for 30 min and at 120 V for 90 min in a Mini-PROTEAN tank (Bio-Rad, Hercules, USA). Samples from different sites were randomly loaded onto gels to account for inter-gel/membrane differences.

The resolved proteins were then electrophoretically transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare, Amersham) at 100 V for 45 min with cooling using a Trans Blot system (Bio-Rad). To normalize the protein loading, membranes were stained with Ponceau S dye (Sigma–Aldrich), and colorimetric images were captured (Bio-Rad ChemiDoc XRS$^+$ System). We incubated the membranes in blocking buffer (5% non-fat dried milk, Tris-buffered saline, 0.1% Tween) for one hour. The membranes were processed for immunodetection with a polyclonal anti-rabbit primary antibody (Sigma Anti-MT1E in TBS-T, 1:200), goat anti-rabbit secondary antibody (biotin in TBS-T, 1:200), Avidin-biotin complex peroxidase staining kit (Thermo Scientific), and metal enhanced DAB substrate kit (Thermo Scientific) according to their respective specifications. The Immunoreactive bands were visualized, imaged and quantified on a Bio-Rad ChemiDoc XRS System. Densitometric analysis of total protein and immunoreactive analysis was performed with ImageLab software, Version 2.0 (Bio-Rad). Total protein signal instead of a single constitutively expressed protein was used to correct for loading differences as in Aldridge et al. (2008).

2.6. Statistical analyses

We pooled data into two groups: WWTW sites (Umbilo, Kingsburgh, Verulam) and unpolluted sites (Unpolluted site 1, Unpolluted site 2) to perform statistical analyses. We compared liver/kidney metal composition among sites using one-way ANOVAs. We performed Spearman/Pearson correlations between HSI and liver MT1E, RSI and kidney MT1E, between liver/kidney MT1E and each element, and between elements that showed significant differences between sites. Histopathological lesion data is presented in Table 1 as total number of lesions, lesion types and percent individuals showing lesions. We performed one-way ANOVAs to compare differences in *N. nana* HSI, RSI, liver MT1E and kidney MT1E between WWTW sites and unpolluted sites. Assumptions of normality and equality of variance were tested using a 1-sample

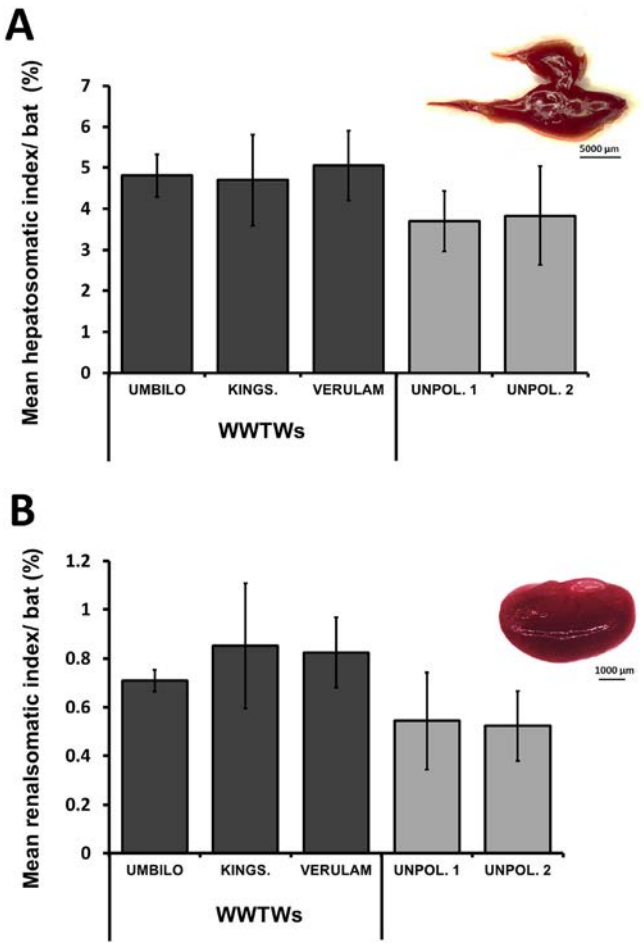

Fig. 4. (A) Mean hepatosomatic index/bat and (B) Mean renalsomatic index/bat of *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park. (U = UWWTW = Umbilo WWTW; V = VWWTW = Verulam WWTW; K = KWWTW = Kingsburgh WWTW; Un. 1 = Unpolluted site 1; Un. 2 = Unpolluted site 2) (P < 0.05 between WWTWs and Unpol. sites). *N* = 24; Bars = ±Std.dev.

Kolmogorov–Smirnov Test and a Levene's Test, respectively. Non-parametric tests were performed where assumptions were not satisfied. All analyses were performed with IBM SPSS 22.0, using alpha of 0.05. In addition, we performed sample size calculations to determine the minimum number of subjects required for an adequate study power of 80% (ClinCalc.com software, ClinCalc LLC). For all variables tested except RSI and elements in the liver and kidney listed below, sample size was low enough for sufficient power of statistical tests. In contrast, the small sample sizes of As, Cd, Hg, Na, and Pb in the liver, and RSI, Cd, Cr, Hg, Ni, Pb in the kidney may result in greater probability of a type-II error; thus statistical analyses using larger sample sizes for these variables may reveal significant differences between bats from WWTWs and unpolluted sites.

3. Results

3.1. Metal composition

Of the 12 metals quantified in the liver, only Fe and Zn were significantly different between sites. Fe and Zn were significantly higher at WWTWs than at the unpolluted sites (Fe: $F_{(1,25)} = 10.363$, $P = 0.004$; Zn: $F_{(1,25)} = 11.944$, $P = 0.002$). (Fig. A.1, Supplementary material). There were no significant correlations between liver MT1E and each of the metals or mineral nutrients.

In the kidney, Cu was significantly higher at WWTWs than at unpolluted sites ($\chi^2_{(1,25)} = 4.27$, $P = 0.039$; Fig. A.2, Supplementary material). Of the mineral nutrients quantified in the kidney, only K was significantly different among sites, with unpolluted sites having significantly higher K than WWTWs ($\chi^2_{(1,25)} = 12.484$, $P = 0.000$) (Fig. A.2, Supplementary material).

3.2. Histological evaluation

We found a higher incidence and variety of histopathological lesions in bats foraging at WWTWs than at unpolluted sites in both the liver and kidney (Table 1). We found five lesion types in the liver and four in the kidney. The organs of bats from the unpolluted sites had minimal lesions (liver: necrosis, vacuolization; kidney: necrosis, infiltration/inflammation) and were generally healthy.

In the liver of bats at WWTWs, we found necrosis, infiltration/inflammation, vacuolization, hyperplasia and atrophy (see Fig. 3 for representative micrographs). In the kidney, we found necrosis, infiltration/inflammation, tubular dilatation and cylinders (see Fig. 3 for representative micrographs). Amongst WWTWs, the liver and kidney showed least damage at the Kingsburgh WWTW (lesions in 67% of individuals) and most damage at the Verulam WWTWs, with 100% of the individuals exhibiting lesions (Table 1).

Only necrosis and infiltration/inflammation occurred in the liver and kidneys of bats at both WWTWs and unpolluted sites. There was a higher percent necrosis and infiltration/inflammation, and therefore a greater severity of these lesions, in the liver and kidneys of individuals from WWTWs compared to unpolluted sites (Fig. 2).

3.3. Hepatosomatic/renalsomatic index (HSI/RSI)

HSI in bats from the WWTW sites was significantly higher than at the unpolluted sites ($F_{(1, 25)} = 12.276$, $P = 0.002$) (Fig. 4A). RSI at the WWTWs was also significantly higher than at the unpolluted sites ($F_{(1, 25)} = 12.722$, $P = 0.002$).

3.4. Metallothionein content

Although liver MT1E in bats from the WWTW sites was significantly lower than at the unpolluted sites ($F_{(1, 25)} = 8.461$, $P = 0.009$; Fig. 5A), the protein fold difference at unpolluted sites was only 1.15 times that of the WWTWs. In addition, liver MT1E content of individuals from both the WWTWs and unpolluted sites were interspersed with each other around the regression line (Fig. 5C).

Kidney MT1E at the WWTWs was significantly lower than the unpolluted sites combined ($F_{(1,25)} = 11.788$, $P = 0.003$; Fig. 5B). However, the protein fold difference at unpolluted sites was only 1.07 times that of the WWTWs. Kidney MT1E content of individuals from the WWTWs and unpolluted sites were again interspersed with each other around the regression line (Fig. 5D). However, individuals from the unpolluted sites were situated slightly above the 95% confidence limits compared to individuals from the WWTWs (Fig. 5D). There were no significant correlations between HSI and liver MT1E, or between liver and kidney MT1E. However, RSI was significantly correlated with kidney MT1E ($P = 0.018$, $r^2 = -0.477$). In addition, there were significant correlations between MT1E and Cu ($P = 0.009$, $r^2 = -0.524$; Fig. 6A)), MT1E and K ($P = 0.002$, $r^2 = 0.600$; (Fig. 6B), and between Cu and K ($P = 0.040$, $r^2 = -0.422$; Fig. 6C).

4. Discussion

We found evidence that pollutant exposure impacts the detoxification organs, the liver and kidney, of *N. nana* foraging at

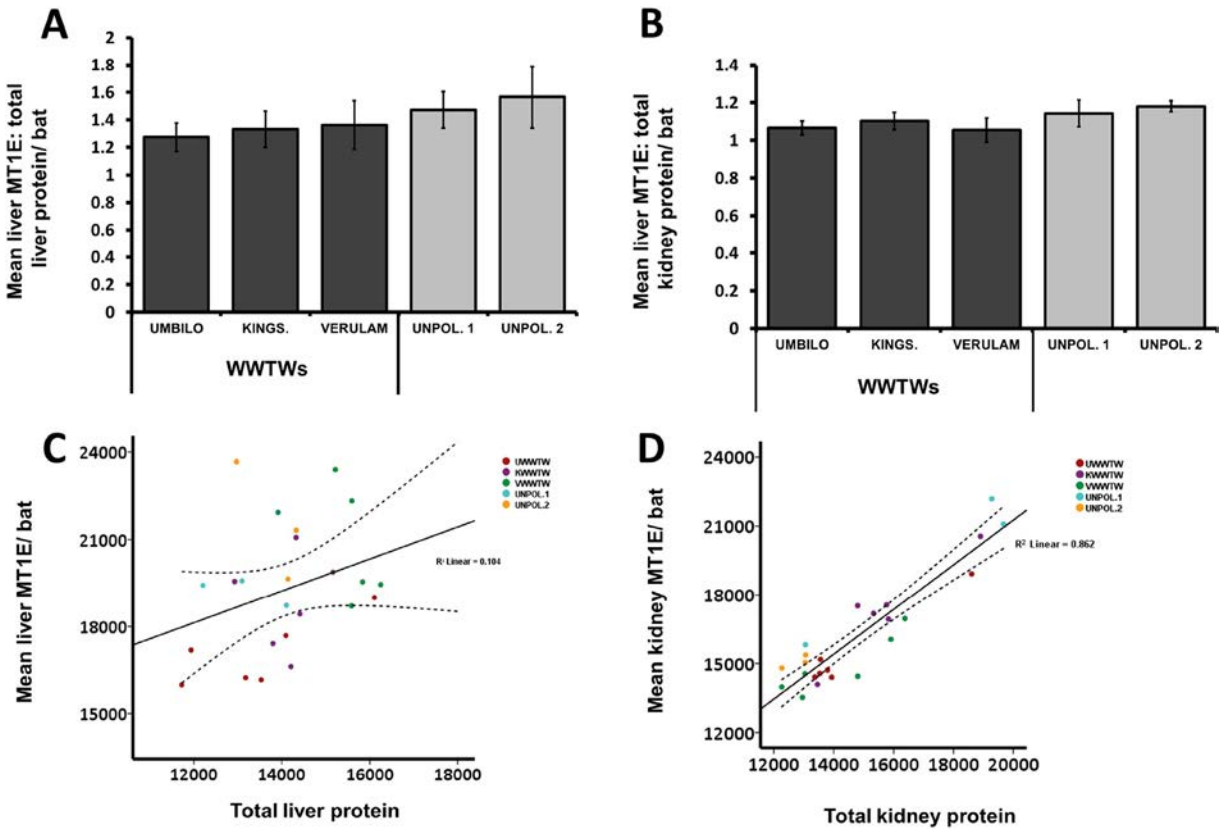

Fig. 5. (A) Liver metalliothionein 1E content, indicated by the mean liver MT1E: total liver protein/bat, and (B) relationship between liver MT1E and total liver protein. (C) Kidney metallothionein 1E content, indicated by the mean kidney MT1E: total liver protein/bat, and (D) relationship between kidney MT1E and total kidney protein in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park. ($P < 0.05$ between WWTWs and Unpol. sites). $N = 24$; Bars = ±Std.dev.

WWTWs. Contrary to our predictions, we did not find significantly higher levels of toxic non-essential metals such as Cd and Pb in the liver and kidneys of WWTW bats. We did, however, find an unexpected potential impact of excess essential metals, namely Fe and Cu in the liver and kidney respectively, as well as lower Zn levels in the liver of WWTW bats. We found a greater extent of histopathological lesions and significantly higher hepatosomatic and renal-somatic indices in WWTW bats, as predicted. We did not, however, find support for our prediction of increased liver and kidney MT1E content in *N. nana* at WWTWs.

Although Fe and Cu are essential metals for living organisms, and have limited bio-availability, at elevated concentrations both Fe (Fraga and Oteiza, 2002) and Cu (Gaetke and Chow, 2003) are toxic and pose a threat to cells and tissues. We previously found Fe levels at the tank sites of both the Umbilo (0.9 ± 0.07 mg L^{-1}) and Verulam (1.3 mg L^{-1}) WWTWs to be significantly higher than unpolluted sites and at river sites located downstream of the WWTWs (Naidoo et al., 2013). Both concentrations were higher than the South African Water Quality Guideline (DWAF, 1996) for human consumption (0–0.1 mg L^{-1}). Indeed, the higher Fe levels we found in the liver of bats in the present study are consistent with previous Fe-induced haematological responses such as an increased haematocrits in *N. nana* at WWTWs (Naidoo et al., 2015). On the other hand, the significantly lower levels of the essential metal Zn in WWTW bats may be of more concern than Zn-toxicity because of its importance in physiological functions (Fox, 1991). More importantly, there is a specific biological interaction between Zn and other metals including Cu and Fe (Fraga and Oteiza, 2002).

Zn acts as an antioxidant and competes with Fe for cellular binding sites (Fraga and Oteiza, 2002). Thus, low Zn levels can cause an increase in membrane and intracellular Fe concentration (Rogers et al., 1987). When binding sites are occupied by Zn rather than Fe, there is reduced iron-mediated oxidation of lipids, proteins, and DNA (Fraga and Oteiza, 2002). Thus, the low Zn and high Fe content in the liver of bats at WWTWs may contribute to liver deterioration. Furthermore, negative effects of Fe are most prominent in the liver (Papanikolaou and Pantopoulos, 2005). Zn also serves a special protective function against Cd-induced tissue damage (Mahran et al., 2011). Therefore, despite the fact that Cd was not significantly higher at WWTW sites than at unpolluted sites, lower Zn levels in these bats may make them more susceptible to the effects of any Cd they are exposed to. Although our earlier work found that Zn levels were significantly higher at WWTW sites than at unpolluted sites (Naidoo et al., 2013), the lower Zn levels observed in *N. nana* at WWTWs may be attributed to exposure to synthetic Zn-chelating agents used to treat high Zn during the wastewater treatment process (Vohra and Kratzer, 1964).

Similarly, we did not predict site differences in mineral elements, however K content in the kidney was significantly lower in *N. nana* at the WWTWs than at unpolluted sites. In contrast, renal Cu content was significantly higher in *N. nana* at WWTWs than at the unpolluted sites. This corresponds with the significantly higher levels of Cu previously quantified at the WWTWs, particularly the Umbilo and Verulam WWTWs, compared to unpolluted sites (Naidoo et al., 2013). Cu can be a potent toxicant because of its redox nature (Grosell et al., 2004). In addition, Cu content was significantly negatively correlated with K content. A primary active process in the kidney is sodium re-absorption which occurs via Na^+/K^+-ATPase pumps (Vander et al., 1994). High levels of Cu have been shown to inhibit Na reabsorption into cells (Grosell et al.,

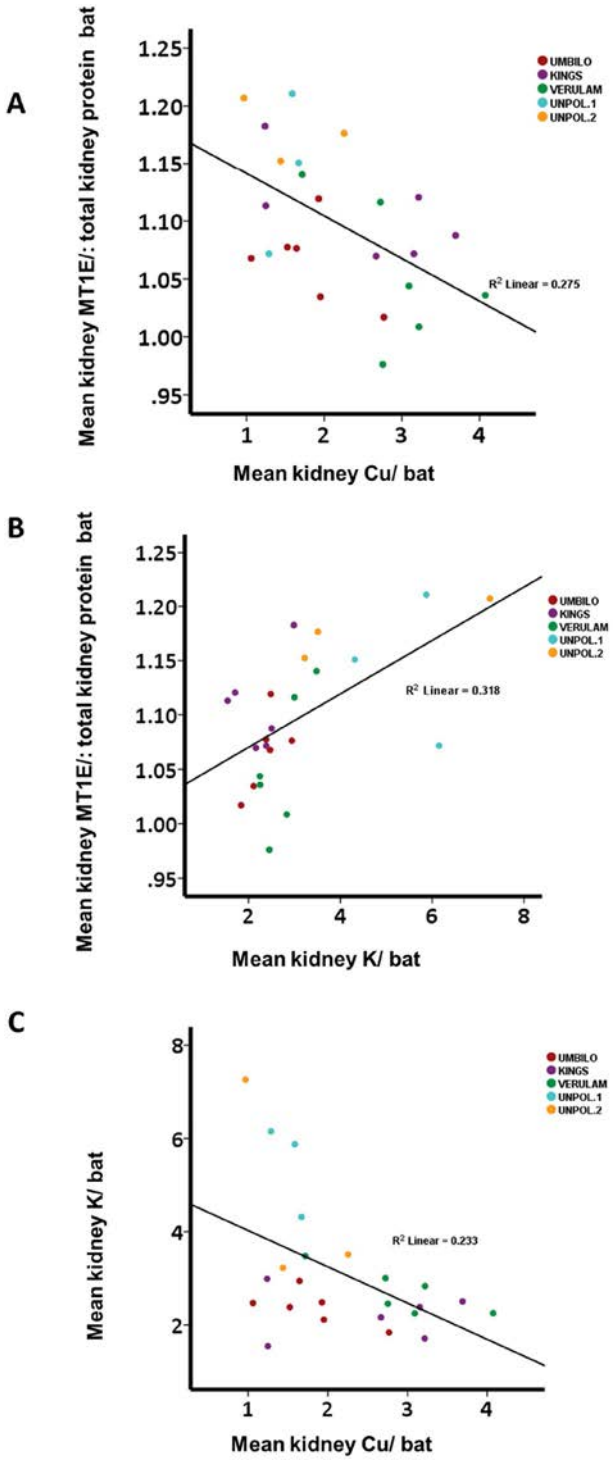

Fig. 6. Relationship between (A) mean kidney MT1E: total kidney protein/bat and mean kidney copper content/bat, (B) mean kidney MT1E: total kidney protein/bat and mean kidney potassium content/bat, and (C) mean kidney potassium content/bat and mean kidney copper content/bat, in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park. ($N = 24$).

2002; Laurén and McDonald, 1985). Inhibited sodium reabsorption, in turn, results in a loss of K (Vander et al., 1994). In addition, the mammalian copper transporter Ctr1, is directly linked to potassium levels in organs including the kidney (Lee et al., 2002). This suggests that the high Cu content in the kidneys of WWTW bats are directly compromising the Na^+/K^+-ATPase pump, causing a decrease in K in the cells. Ultimately, this may contribute to sodium deficiency and potassium deficiency linked disorders in bats at WWTWs. However, we used SEM-EDS to provide a measure of metal differences between bats from WWTWs and unpolluted sites. SEM-EDS is a semi-quantitative method, which serves to detect differences between groups (or individuals). It is not a technique that quantifies the total amount of metals. To understand the extent of disturbance in ionic regulation, future work should use quantitative techniques such as AAS (Atomic Absorption Spectroscopy) or ICP (Inductively Coupled Plasma Mass Spectrometry). However, our previous work highlighted high metal loads in the liver and kidney of bats foraging at these WWTW sites, and the samples were indeed analysed by ICP-OES (Naidoo et al., 2013). The high Cu levels detected in the current study were also highlighted in our previous study.

Although toxic non-essential metals like Pb and Cd were not significantly higher in *N. nana* at WWTWs, elevated Fe and Cu concentrations in liver and kidney of bats at WWTWs may be causing sub-lethal effects in these detoxification organs. Furthermore, the high variability in As, Cd, Hg, Na, Pb (in the liver) and RSI, Cd, Cr, Hg, Ni, Pb (in the kidney) data probably resulted in low statistical power in detecting differences between bats from WWTWs and unpolluted sites. We found a greater extent of tissue lesions indicative of histological damage in the liver and kidney of *N. nana* at WWTWs. Necrosis and infiltration/inflammation were found in liver and kidney of bats at all sites. More specifically, incidence and variety of histopathological lesions were higher in both the liver and kidney of bats foraging at WWTWs than at unpolluted sites. The extent of the both the necrosis and infiltration/inflammation was more severe in WWTW bats, with a greater percent of tissue area occupied by the lesion. When oxidative stress from pollutants causes DNA damage, tissue lesions such as necrosis and inflammation occur. Cell-cycle arrest, as seen in necrosis, has been observed in response to not only metals in laboratory experiments, but also in response to environmental landfill leachates (Damek-Poprawa and Sawicka-Kapusta, 2003; Sanchez-Chardi et al., 2008, 2009). Landfill leachates may be similar to the pollutant exposure at WWTWs in that contamination is by a mixture of pollutants from decomposing solid waste. For instance, wood mice (*Apodemus sylvaticus*), and greater white-toothed shrews (*C. russula*) collected from the Garraf landfill in Spain, showed severe histopathological alterations in both the liver and kidney (Sánchez-Chardi et al., 2009). An increase in tissue lesions such as necrosis and inflammation may lead to sub-optimal organ function and therefore negatively impact the general health of the bats. Importantly, these lesions may ultimately result in tumour formation, cancers and other diseases which may reduce lifespan (Pastink et al., 2001; Van Gent et al., 2001).

Additionally, hyperplasia, vacuolization and atrophy in the liver were found only in WWTW bats. Vacuolization is of particular note, because its occurrence in the liver suggests a lipid/water disturbance which is linked to metabolic disturbance (Pereira et al., 2006). The potential metabolic disturbance in the bats was also supported by enlarged kidneys, as discussed below. In the kidney, tubular dilatation and cylinders were found only in WWTW bats. Both lesions may be indicative of early nephrotoxicity and therefore bats foraging at WWTWs may be at a higher risk for chronic kidney disease (Dakrory et al., 2015). The greatest extent of histopathological damage occurred in both the liver and kidney of bats at the Verulam WWTWs, with 100% of the individuals exhibiting lesions. It is perhaps notable that Verulam WWTWs also had higher Cu levels than other WWTWs. Excess Cu results in the depletion of glutathione, an important antioxidant, which enhances cellular toxicity (Chen et al., 2006). In mice, Cu ions cause degeneration of renal tubules, which can lead to inflammation in renal tissues (Chen et al., 2006).

Toxicological effects on a whole organ level are commonly assessed using organ weight data, especially hepatic and renal weight (Michael et al., 2007; Tête et al., 2013). We found significantly higher hepatosomatic and higher renalsomatic indices in WWTW bats. Increased hepatosomatic/renalsomatic indices in response to pollutants suggest tissue damage such as hepatocellular or tubular hypertrophy (increased cell size) or hyperplasia (increased cell proliferation) which may increase the organ size (Hall et al., 2012). Therefore, organ indices must be interpreted in conjunction with histopathological data to determine the cause of organ enlargement. Histological analyses did not reveal hypertrophy in the liver or kidney of bats at WWTWs. However, we found evidence of hyperplasia in the liver of bats at the Verulam WWTWs, and these bats also had significantly higher Fe levels in the liver. Thus, hyperplasia in response to Fe accumulation in the liver may be contributing to the higher hepatosomatic index at WWTWs. Because there was no evidence of histopathological change associated with size increases in the kidney, the higher renalsomatic index of bats at WWTWs may be indicative of a pollutant exposure effect other than tissue damage from metals such as metabolic disturbance. Kidney weight is highly dependent on changes in metabolism (Liro, 1985). In rodent species, for example, unfavourable external conditions can cause metabolism to intensify, leading to an increase in the relative weights of kidneys (Schwarz et al., 1964).

Because we predicted that the detoxification organs of bats at WWTWs would have higher levels of toxic non-essential metals, we expected a higher MT1E content in response. However, there was no notable protein-fold difference in liver MT1E among sites. Although there was a very low protein fold difference, kidney MT1E was in fact, significantly lower at WWTWs than at unpolluted sites. There are several potential reasons why this trend was observed. Firstly, toxic non-essential metals like Cd and Pb are strong MT1E inducers. MT1E is also induced by Cu, which was significantly higher in the kidney of WWTW bats. However, this increasing effect of Cu on MT1E in bats at WWTWs may be confounded by the high levels of other metals which induce MT1E, particularly Zn (Kelly et al., 1996), which was higher at unpolluted sites. Thus, the seemingly lower MT1E at unpolluted sites may be, in part, due to the lower Zn levels in these bats. In addition, MT1 and MT2 specifically protect against Zn deficiency by serving as reservoirs (Kelly et al., 1996). MT-bound Zn is thus stored and used for physiological functions requiring Zn, when Zn availability is low. Thus, MT may be decreased at WWTWs because they are being utilized to compensate for the lower Zn levels in bats at WWTWs than from unpolluted sites.

Secondly, there are various isoforms of MT (MT-I, MT-II, MT-III and MT-IV) (Thirumoorthy et al., 2011). Although metal-binding and detoxification is characteristic of the MT1E isoform we quantified, other MT isoforms may be involved and being upregulated in WWTW bats (Sakulsak, 2012). Further, metallothionein levels may also be influenced by other factors including hormones such as glucocorticoids (Quaife et al., 1986). Another possible reason why MT1E in bats at WWTWs is not upregulated, is that the bats receive multiple exposures to varying metal concentrations and are exposed to a combination of them (Dai et al., 2013). Thus, metallothionein regulation in wild bat populations may not correlate with patterns observed in laboratory studies with controlled metal exposures.

Finally, the synthesis and extent of metallothionein response differs widely among species (Henry et al., 1994). Internal metal concentrations and metallothionein levels in sympatric small mammal species along the same pollution gradient showed species-specific patterns (Fritsch et al., 2010). Low metal accumulation was associated with high metallothionein in wood mice (*A. sylvaticus*), but with low metallothionein in bank voles (*Myodes glareolus*). In common (*Sorex araneus*) and pygmy (*S. minutus*) shrews, elevated metal accumulation resulted in a sharp increase of metallothionein (Fritsch et al., 2010). Among vespertilionid bats, the highest metallothionein levels were found in *Pipistrellus pipistrellus*, an aquatic-insect-foraging species (Pikula et al., 2010). Notably, Pikula et al. (2010) found a negative correlation between metallothionein and Pb levels, and conclude that the use of MT levels as a biomarker of exposure to heavy metals may not be so straightforward in vespertilionid bats.

To conclude, increased pollutant exposure at WWTWs disrupts the balance of essential metals and mineral nutrients in the liver and kidneys of *N. nana* foraging at WWTWs. Although innate physiological mechanisms such as metallothionein may offer protection against tissue damage, the regulation of these mechanisms are complex and may themselves be compromised at WWTWs. Whole organ effects, represented by increased hepatosomatic and renalsomatic indices are evident in *N. nana* at WWTWs and may be indicative of metabolic disturbance. This is accompanied by additional organ damage indicated by histopathological damage within the liver and kidney tissues. Although these lesions may be caused by high levels of metals such as Cu at WWTWs, they may also result from organic pollutants that the bats are exposed to during the wastewater treatment process. In fact, the physiological effects noted here, are not specifically restricted to metals. In addition to metals, wastewater contains a range of contaminants including pharmaceuticals and personal care products which may contribute to these effects (Gibson et al., 2005; Jones et al., 2007). Thus, while the effect of metal exposure on bats is of great concern (Zukal et al., 2015), the combination of metals with other pollutants, especially at WWTWs poses a serious threat to urban exploiter bats such as *N. nana*. Future work should therefore quantify both metals and organic pollutants that affect bats during the wastewater treatment process. Taken together, our results show that foraging at WWTWs affects the ecology (Naidoo et al., 2013), haematology/genotoxicity (Naidoo et al., 2015) and detoxification organs (this study) of *N. nana*. It is likely that chronic health problems related to sublethal pollutant exposure increases mortality in the long-term. We are currently investigating effects of foraging at WWTWs on reproductive organs and sex hormones in our laboratories. Ultimately, the fitness of urban *N. nana* populations exploiting WWTWs may be affected.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.09.056.

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Supplementary material: Appendix A

Pollutant exposure at wastewater treatment works affects the detoxification organs of an urban adapter, the Banana Bat

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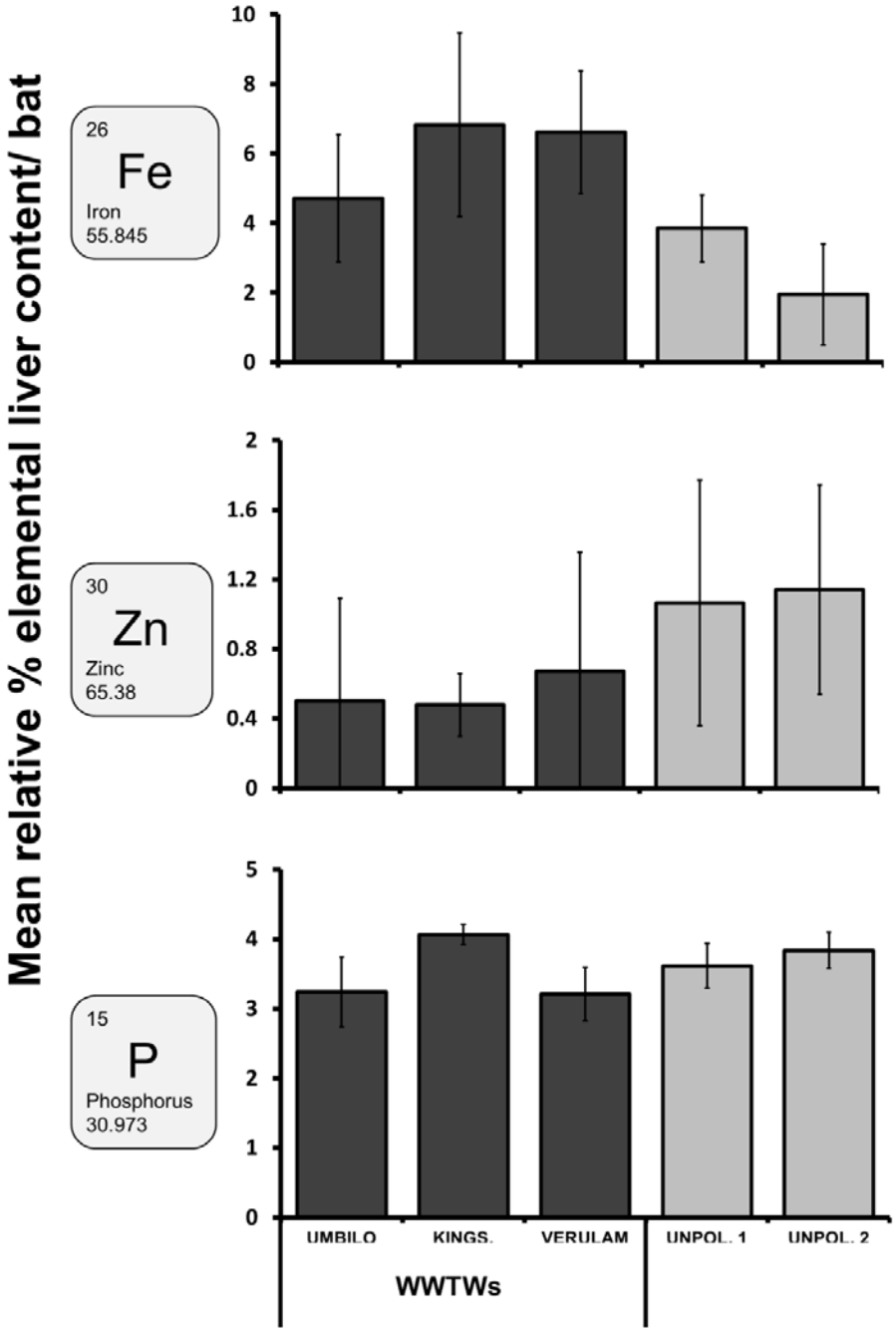

Fig. A.1. Mean relative % elemental liver content / bat for elements (Fe, Zn and P) with significant site differences in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park. ($P < 0.05$ between WWTWs and Unpol. sites). $N = 24$; Bars = ± Std.dev.

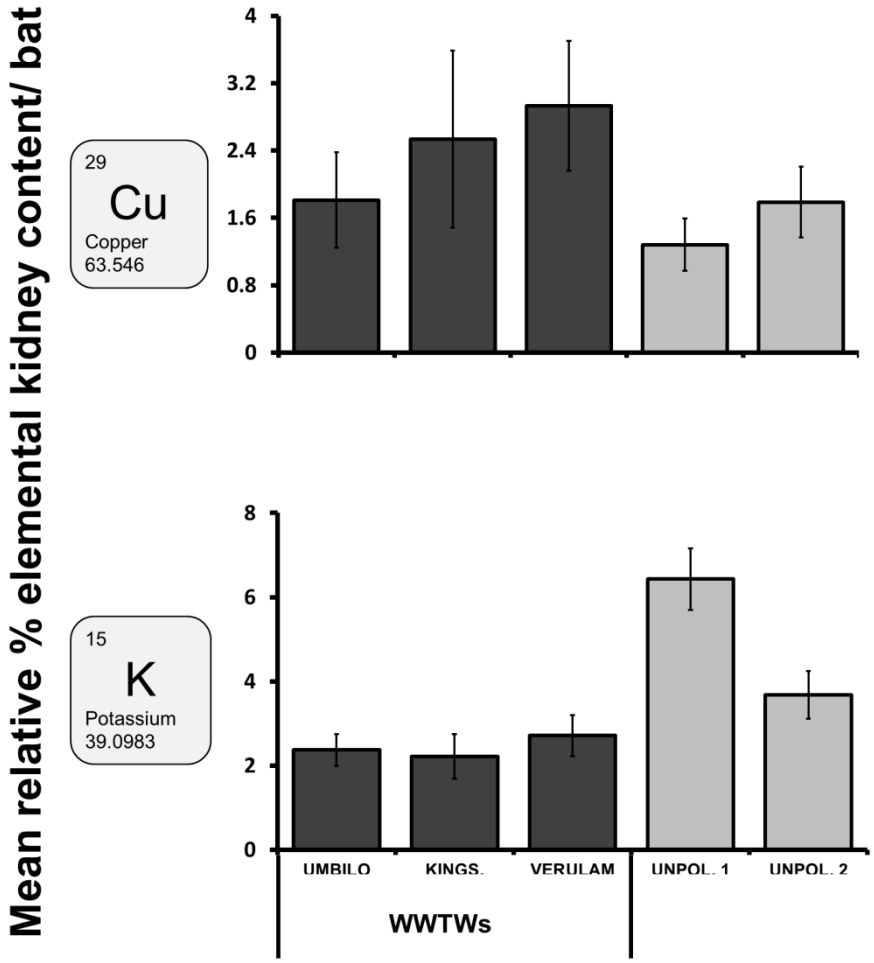

Fig. A.2. Mean relative % elemental kidney content / bat for elements (Cu and K) with significant site differences in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites 1 and 2 at Umdoni Park. ($P < 0.05$ between WWTWs and Unpol. sites). $N = 24$; Bars = ± Std.dev.

Chapter 5

Sex and the city: Reproductive system alterations in an urban adapter, the banana bat, exposed to endocrine-disrupting chemicals at wastewater treatment works

CHAPTER 5:
Sex and the city: Reproductive system alterations in an urban adapter, the banana bat, exposed to endocrine-disrupting chemicals at wastewater treatment works

5.1. Abstract

The reproductive system may be particularly vulnerable to the negative effects of pollutants, particularly endocrine-disrupting chemicals (EDC), which occur in high concentrations at WWTWs. *Neoromicia nana,* the banana bat, is an urban exploiter that forages extensively on pollution-tolerant insect prey at wastewater treatment works (WWTWs) within the urban landscape. We have previously found high metal levels in different tissues, and sub-lethal physiological effects in haematological parameters and detoxification organs of *N. nana* at these sites. We investigated the reproductive system of male *N. nana* foraging at WWTWs in the context of four hypotheses of EDC effect: (i) We quantified the concentration of plasma testosterone - the primary male sex hormone - and found significantly lower levels in WWTW bats (ii) We measured baculum morphometric parameters to investigate effects of early-life exposure, and found no significant differences (iii) We calculated gonadosomatic indices (GSI) to investigate whole organ effects on testes, and found no significant differences between sites, and iv) We calculated body condition indices (BCI), to compare body condition as a general indication of male quality. WWTW bats had significantly lower BCI than bats from unpolluted sites. Taken together, our results suggest that alterations to the endocrine system and body condition may impact reproduction, with serious negative consequences for evolutionary fitness of males in urban *N. nana* populations at WWTWs.

Keywords: *Neoromicia nana*, Wastewater treatment works, Endocrine-disrupting chemical, Testosterone, Baculum, Testis, Gonadosomatic index, Body condition index.

5.2. Introduction

Wastewater treatment works (WWTWs) are common and often permanent fixtures scattered across urban landscapes. They provide an essential service to human populations, however, in serving to remove contaminants from reclaimed water, they themselves act as a source of pollution to the

wildlife exposed to them. WWTW tanks contain an extensive list of contaminants, many of which are not completely removed, and thus remain exposed during treatment and in the treated effluent discharged to rivers (Auriol et al., 2006).

Pollutants not only cause physical damage to cells, tissue and organs, but also interfere with the homeostatic functioning of physiological systems. The endocrine system of animals for instance, is particularly vulnerable to the negative effects of pollutants (Colborn et al., 1993). Hormones under the control of the endocrine system are constantly maintained in balance. However, endocrine disrupting chemicals (EDCs) bind to hormone receptors and modulate, mimic, enhance, or inhibit the action of endogenous hormones (Tyler et al., 1998). Because humans produce and excrete large quantities of natural and synthetic hormones from oral contraceptive use, hormone replacement therapy and other medicinal purposes, WWTWs in particular, receive high quantities of these estrogenic and androgenic compounds (Huang and Sedlak, 2001). In addition, industrial waste received, contains EDCs in the form of organochlorine pesticides, specific metals including cadmium and lead, polychlorinated biphenyls (PCBs), dioxin-like chemicals, bisphenol-A, alkylphenolic chemicals, inclozolin fungicide, tributyl tin and plasticizer phthalates (Tyler et al., 1998). The efficiency of EDC removal from wastewater varies with the hydrophobicity and molecule size of the specific chemical (Nakada et al., 2006). Thus, effluent may contain high levels of certain chemicals even after treatment (Janex-Habibi et al., 2009). For instance, treated wastewater effluent contains steroidal estrogens (Routledge et al., 1998) and alkylphenols (Routledge and Sumpter, 1996) in quantities sufficient to affect testis growth in fish.

The EDCs found in wastewater can severely alter the reproductive physiology and behaviour of the resident wildlife exposed to them. Exposure can be directly with the water or indirectly via predator-prey interactions. For example, industrial and household waste received at WWTWs undergoes treatment in open-top sludge tanks, producing a characteristic proliferation of pollution-tolerant chironomid midge swarms (Boonstra et al., 2009). The midges retain pollutants, including metals and other EDCs (Park et al., 2009) that they are exposed to, within their body tissue (Hare et al., 1992; Krantzberg and Stokes, 1990). Park et al. (2009) quantified EDCs such as 17a-ethinylestradiol and butylated hydroxy aniline, and the estimated daily exposure rate for bats feeding on aerial invertebrates at sewage filter beds. The estimated EDC exposure for bats was nearly equivalent to levels which cause harmful effects in male European starlings (*Sturnus vulgaris*) (Markman et al., 2008; Park et al., 2009). Numerous cases of intersex fish (Tyler and Jobling, 2008), frogs (Cevasco et al., 2008), and reptiles (Milnes, 2005) that were exposed to EDCs

have been observed. In addition, EDCs elicit various reproductive system effects in crustaceans, including abnormal development of secondary sexual characters (Rodriguez et al., 2007).

Reproductive system effects from EDCs are not, however, confined to aquatic and semi-aquatic taxa. In birds, egg-shell thinning (Blus et al., 1997) and altered song patterns (Markman et al., 2008) have been documented in several species. A growing body of evidence is now highlighting the negative effects of EDCs on mammalian reproduction, from porpoises to humans (Pelch et al., 2010). These effects are primarily produced in response to the hormone-altering actions of EDCs. Because the reproductive system is governed by hormones, the sex organs are particularly affected. For instance, disruptions to testosterone levels in males may affect testis size (Garamszegi et al., 2005). The development of the baculum, or os-penis during pre-natal and early life is also mediated by androgens, especially testosterone (Yonezawa et al., 2011). In East Greenland polar bears (*Ursus maritimus*), exposure to xenoendocrine pollutants through the consumption of contaminated seal prey, reduced genitalia size and baculum bone density (Sonne et al., 2006). Although testis size was not affected, baculum length was positively associated with PCB congeners in wild mink (*Neovison vison*) from Sweden (Persson and Magnusson, 2015). Alterations to sex organs and sex hormones will ultimately affect an individual's ability to acquire mates and reproduce, and thus impact fitness. EDCs may thus alter hormone levels, anatomy, physiology, behaviour and fitness (Pelch et al., 2011). Furthermore, they can act at very low doses (Pelch et al., 2011).

The banana bat, *Neoromicia nana* (family Vespertilionidae), is an insect-eating bat and urban adapter that profits from resources provided by humans within anthropogenic landscapes (Jung and Kalko, 2011). *N. nana* typically roosts in rolled-up banana leaves ((*Musa* and *Ensete* spp.; LaVal and LaVal, 1977) yet will also roost in thatched roofs of houses (O'Shea 1980; Monadjem and Fahr, 2007), and in curled leaves of strelitzia (*Strelitzia caudate* and *S. nicolaii*) planted in private residential gardens (M.C. Schoeman, unpubl. data). In addition to this abundant availability of anthropogenic roost sites, *N. nana* exploits WWTWs within the urban landscape. We previously found that *N. nana* was the dominant animalivorous bat species foraging at wastewater-polluted sites (Naidoo et al., 2011). Moreover, chironomid midges were the most abundant prey type in the diet of *N. nana* at wastewater-polluted sites, compared to a diverse insect diet in *N. nana* at unpolluted sites (Naidoo et al., 2013; Schoeman and Jacobs 2011). However, *N. nana* at WWTWs exhibited higher levels of metals in the tissue (Naidoo et al., 2013), and sub-lethal haematological and genotoxic responses, including significantly lower antioxidant capacity and significantly higher levels of DNA damage and haematocrits than bats from unpolluted sites (Naidoo et al., 2015). In addition, we found sub-lethal damage to the organs responsible for detoxifying pollutants.

Differences in essential metal (Fe and Zn) content in the liver, and in Cu and one mineral nutrient (K) in the kidneys were accompanied by significant increases in liver and kidney size and greater extent of histopathological lesions in WWTW bats (Naidoo et al., 2016). It is therefore likely that *N. nana* foraging at WWTWs also exhibits effects from EDC exposure.

The aim of this study was to therefore to investigate the reproductive system of male *N. nana* foraging at WWTWs in the context of four hypotheses of EDC effect: (i) We quantified the concentration of circulating testosterone, the primary male sex hormone (ii) We measured baculum morphometric parameters to investigate whether early-life exposure to pollutants affects baculum development, (iii) We calculated gonadosomatic indices (GSI) to investigate whole organ effects on testes, and (iv) We calculated body condition indices (BCI), to compare body condition as a general indication of male quality and fitness. We predicted that, compared to male *N. nana* foraging at unpolluted sites, *N. nana* foraging at WWTWs should have (i) lower plasma testosterone levels, (ii) smaller bacula, (iii) lower GSI, and (iv) lower BCI.

5.3. Methods

5.3.1. Sample Collection

We used mist nets and harp traps to capture *N. nana* bats at three WWTW sites and at two unpolluted reference sites. We sampled *N. nana* at sludge tanks that contain high levels of wastewater –associated metals (lead, cadmium, chromium, nickel, copper, zinc and iron; Naidoo et al., 2013) in the Umbilo Wastewater Works (S29º50.44’; E30º53.31’), the Verulam Wastewater Works (S29º38.38’; E31º03.49’), and the Kingsburgh Wastewater Works (S30º04.29’; E30º51.26’) located within Durban, South Africa (S29°58’; E30°57’) (see Naidoo et al. (2015) for map). We captured *N. nana* bats at two unpolluted reference sites in the forest of Umdoni Park (S30º41.15’; E30º23.35’), located in Pennington, about 80 km south of Durban. There are no WWTWs located in the immediate vicinity of the park, with the closest WWTW situated > 8 km away. Furthermore, individual turnover between unpolluted sites and contamination from the nearest WWTW was unlikely because of the relatively small home range of *N. nana* (~ 300 m from the roost (LaVal and LaVal, 1977)).

Captured bats were identified to species using a taxonomic key (Monadjem et al., 2010) and only male adult, *N. nana* were kept for analyses. Life-stage (juvenile or adult) was assessed from the

presence of cartilaginous epiphyseal plates (Anthony, 1988). We performed sampling during the summer (January - March 2013), before mating, which in *N. nana* occurs during late April to August (Van der Merwe and Stirneman, 2007). However, to account for any differences in testes size, between the January to March sampling period, we sampled bats from the reference sites at the beginning and end of the sampling period. In addition, we only used males with a similar degree of visible descended testicular state. The bats were humanely euthanized, as approved by the University of KwaZulu-Natal Animal Ethics Committee (Reference: 014/15/Animal). They were sacrificed at the same time every day, to remove the potential effect of diurnal fluctuations in plasma testosterone levels (Maurel et al., 1981).

5.3.2. Testosterone Concentration

Whole peripheral blood was collected from each bat and allowed to coagulate. We centrifuged the samples at 8050 × *g* for 10 min and stored the supernatant at ~ 80 ° C until further analysis. We quantified plasma testosterone concentrations in the supernatant using a colorimetric, competitive Testosterone ELISA kit (Enzo Life Sciences Inc.). We diluted 5µL of sample plasma with standard diluent (sample: diluent in a 1:20 ratio) and added 50µL of testosterone ELISA antibody (mouse monoclonal) into each well of a goat anti-mouse IgG coated microtiter plate. We incubated the plate at room temperature on a plate shaker for 1 hour at 500rpm. 50µL of testosterone ELISA conjugate (alkaline phosphatase) was added into each well and the plate was again incubated at room temperature on a plate shaker for 1 hour at 500rpm. We performed three washes on the plate with a Tris-buffered saline buffer. 200µL of p-nitrophenyl phosphate substrate was added to each well and incubated at 37°C for 1 hour. The optical density of the plate was read at 405nm in a BioTek PowerWave XS multiwell plate reader. Standard curves from the assay were used to calculate the total plasma testosterone concentration (ng/mL) in each sample. To minimize inter-assay variation, all samples were analysed at the same time. The intra-assay coefficient of variance was 21.6% for the optical densities quantified.

5.3.3. Baculum Morphometrics

We hydrated the penis from each bat in distilled water and then placed the penis in a 5% KOH solution with alizarin stain for 40 minutes, following Kearney et al. (2002). The baculum was then dissected from the penis using insect pins, fine forceps and a dissecting microscope. We cleared the

dissected baculum in glycerine solutions of 20%, 40%, 60% and 80% for a period of 24 hours in each concentration. The baculum was then placed in 100% glycerine with a thymol crystal to prevent fungal growth until imaging. We viewed and digitally captured images of the bacula using a camera attached to a fluorescence microscope (Nikon Eclipse E400 microscope; magnification = 400 ×). We used phase contrast microscopy to enhance the contrast of the image because of the translucent nature of the baculum. We measured the total baculum length (TBL), total baculum width (TBW), shaft length (SL), shaft width (SW), gap length (GL), gap width (GW), side view length (SVL) and side view width (SVW) using NIS-Elements D (Nikon) software. All measurements were converted to an index relative to forearm length of the bat to account for body size differences.

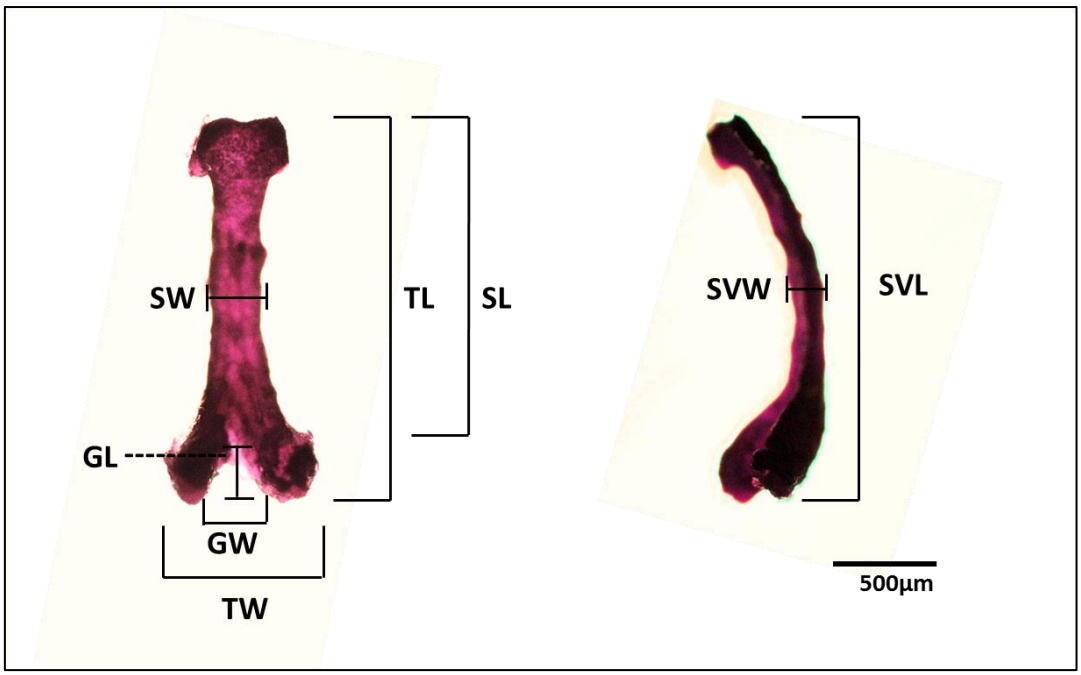

Fig 1. Baculum measurements were total length (TL), total width (TW), shaft length (SL), shaft width (SW), gap length (GL), gap width (GW), side view length (SVL) and side view width (SVW).

5.3.4. Gonadosomatic Indices (GSI)

The testes were dissected from each bat, kept moist to prevent dehydration and immediately weighed on a three decimal balance (wet weight (WW); to nearest 0.001 g). The gonadosomatic index was calculated as a percentage of the average testis weight relative to the total body weight of each individual (average testis weight/total body weight x 100) (Sellers et al., 2007).

5.3.5. Body Condition (BCI)

We measured the forearm and body length (to nearest 0.1 mm), and body mass (to nearest 0.5 g) of each *N. nana* collected. To evaluate body condition, we calculated both the ratio and residual index for two measures of body size, forearm length and body length. We calculated standard ratio body condition indices as BCI-1 (body mass/ forearm length) and BCI-2 (body mass/ total body length). Although BCI-2 is employed to calculate body condition in most taxa, BCI-1 is more commonly used in bats because forearm length is a good indication of size (Speakman and Racey, 1986). Schulte-Hostedde et al., (2005) tested multiple methods and found that the residuals from an ordinary least squares (OLS) regression as indices of body condition, satisfy critical assumptions and correlate well with the proportion of mass associated with energy reserve. We therefore used the residuals from OLS regressions of body mass against forearm length (BCI-3) and of body mass against total body length (BCI-4) as additional measures of condition.

5.3.6. Statistical Analyses

We pooled data into two groups: WWTW sites (Umbilo, Kingsburgh, Verulam) and unpolluted sites (Unpolluted site 1, Unpolluted site 2) because sample size per site was too low to perform robust statistical analyses (as discussed in Naidoo et al., 2016). We compared testosterone concentration, baculum morphometric parameters, GSI and all measures of BCI between WWTWs and unpolluted sites using one-way ANOVAs. We performed a MANOVA between WWTWs and unpolluted sites using the baculum morphometric parameters as dependent variables. We performed Spearman/ Pearson correlations between testosterone concentration and each of the baculum morphometric parameters, GSI and BCI measures, between each of the baculum morphometric parameters versus GSI and BCI measures, and between GSI and BCI measures. Assumptions of normality and equality of variance were tested using a 1-sample Kolmogorov-Smirnov Test and a Levene's Test, respectively. Non-parametric tests were performed where assumptions were not satisfied. All analyses were performed with IBM SPSS 22.0, using alpha of 0.05**.** We also performed sample size calculations to determine the minimum number of subjects required for an adequate study power of 80% (ClinCalc.com software, ClinCalc LLC). For testosterone concentration, BCI-1 and BCI-2, sample size was low enough for sufficient power of statistical tests. However, for GSI, BCI-3, BCI-4 and the baculum morphometric parameters, the large overlap in values between sites suggests that

larger sample sizes for these variables may reveal significant differences between bats from WWTWs and unpolluted sites.

5.4. Results

5.4.1. Testosterone Concentration

Testosterone in bats from the WWTW sites was significantly lower than at the unpolluted sites ($F_{(1, 23)} = 50.295$, $P = 0.000$) (Fig. 2). There was a notably large hormone fold difference where testosterone in bats at WWTWs was 25.89 ± 5.99 (mean ± std. dev.) times lower than that of the unpolluted sites. Testosterone was significantly positively correlated with BCI-1 ($P = 0.039$, $r^2 = 0.433$) and BCI-2 ($P = 0.014$, $r^2 = 0.506$).

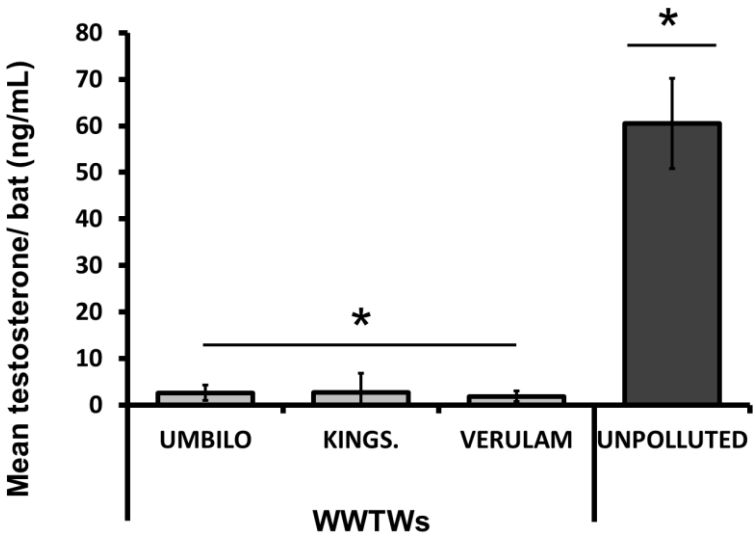

Fig. 2. Mean testosterone concentration in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites at Umdoni Park. (* indicates significant differences between the sites at the $P < 0.05$ level). $N = 23$; Bars = Mean ± Std. dev.

5.4.2. Baculum Morphometrics

There were no significant differences in baculum morphometric parameters (TL, TW, SL, SW, GL, GW, SVL and SVW; all $P > 0.05$) between bats from the WWTWs and unpolluted sites. Means ± Std. dev. for the eight parameters are presented in Table 1. The MANOVA was not statistically significant for baculum morphometric parameters based on site ($F_{(7, 14)} = 1.75$, $P = 0.172$; Wilk's $\Lambda = 0.5$, partial $\eta^2 = 0.5$). There were no significant correlations between baculum morphometric parameters and testosterone concentration, GSI or BCI measures.

Table 1. Baculum measurements (total length (TL), total width (TW), shaft length (SL), shaft width (SW), gap length (GL), gap width (GW), side view length (SVL) and side view width (SVW); Mean (μm) ± Std. dev.)) of *N. nana* at the Umbilo (UWWTW), Kingsburgh (KWWTW), and Verulam (VWWTW) WWTWs and unpolluted sites (UNPOL.) at Umdoni Park.

BACULUM PARAMETERS	SITE			
	UWWTW	KWWTW	VWWTW	UNPOL.
TL	30.26 ± 4.01	29.52 ± 1.62	31.05 ± 2.87	30.61 ± 3.38
TW	10.36 ± 1.49	10.02 ± 1.26	10.66 ± 1.70	10.01 ± 1.36
SL	23.49 ± 3.57	23.10 ± 1.00	24.20 ± 2.31	22.61 ± 1.29
SW	5.48 ± 0.70	5.13 ± 0.34	5.43 ± 0.90	5.04 ± 0.33
GL	6.30 ± 1.12	5.52 ± 0.95	6.61 ± 0.63	7.31 ± 1.77
GW	5.57 ± 1.19	5.15 ± 0.98	5.53 ± 0.70	4.62 ± 0.50
SVL	4.46 ± 0.80	3.78 ± 0.69	3.83 ± 0.70	3.73 ± 0.47
SVW	30.19 ± 3.27	30.49 ± 0.68	34.76 ± 2.69	31.48 ± 1.61

5.4.3. Gonadosomatic Indices (GSI)

There was no significant difference in the gonadosomatic index between bats from the WWTWs and the unpolluted sites ($F_{(1, 23)} = 0.151$, $P = 0.701$) (Fig. 3). There were no significant correlations between GSI and testosterone concentration, baculum morphometric parameters or BCI measures (all $p>0.05$).

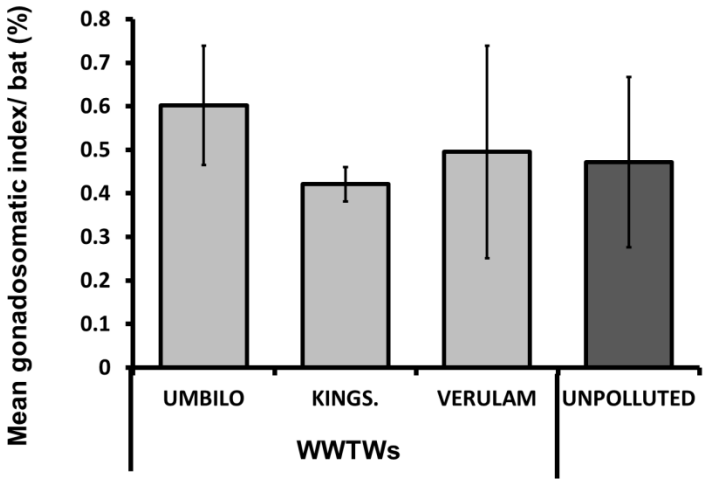

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Fig. 3. Mean gonadosomatic index in *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites at Umdoni Park. $N = 23$; Bars = Mean ± Std. dev.

5.4.4. Body Condition (BCI)

Bats from the WWTWs had significantly lower BCI-1 ($F_{(1, 23)} = 14.153$, $P = 0.001$), BCI-2 ($F_{(1, 23)} = 17.573$, $P = 0.000$) (Fig. 4 A), BCI-3 ($F_{(1, 23)} = 13.117$, $P = 0.002$) and BCI-4 ($F_{(1, 23)} = 10.311$, $P = 0.004$) (Fig. 4 B) than bats from the unpolluted sites. In addition, there were significant positive correlations between testosterone concentration and BCI-1 ($P = 0.039$, $r^2 = 0.433$) (Fig. 4 C), and between testosterone concentration and BCI-2 ($P = 0.014$, $r^2 = 0.506$) (Fig. 4 D).

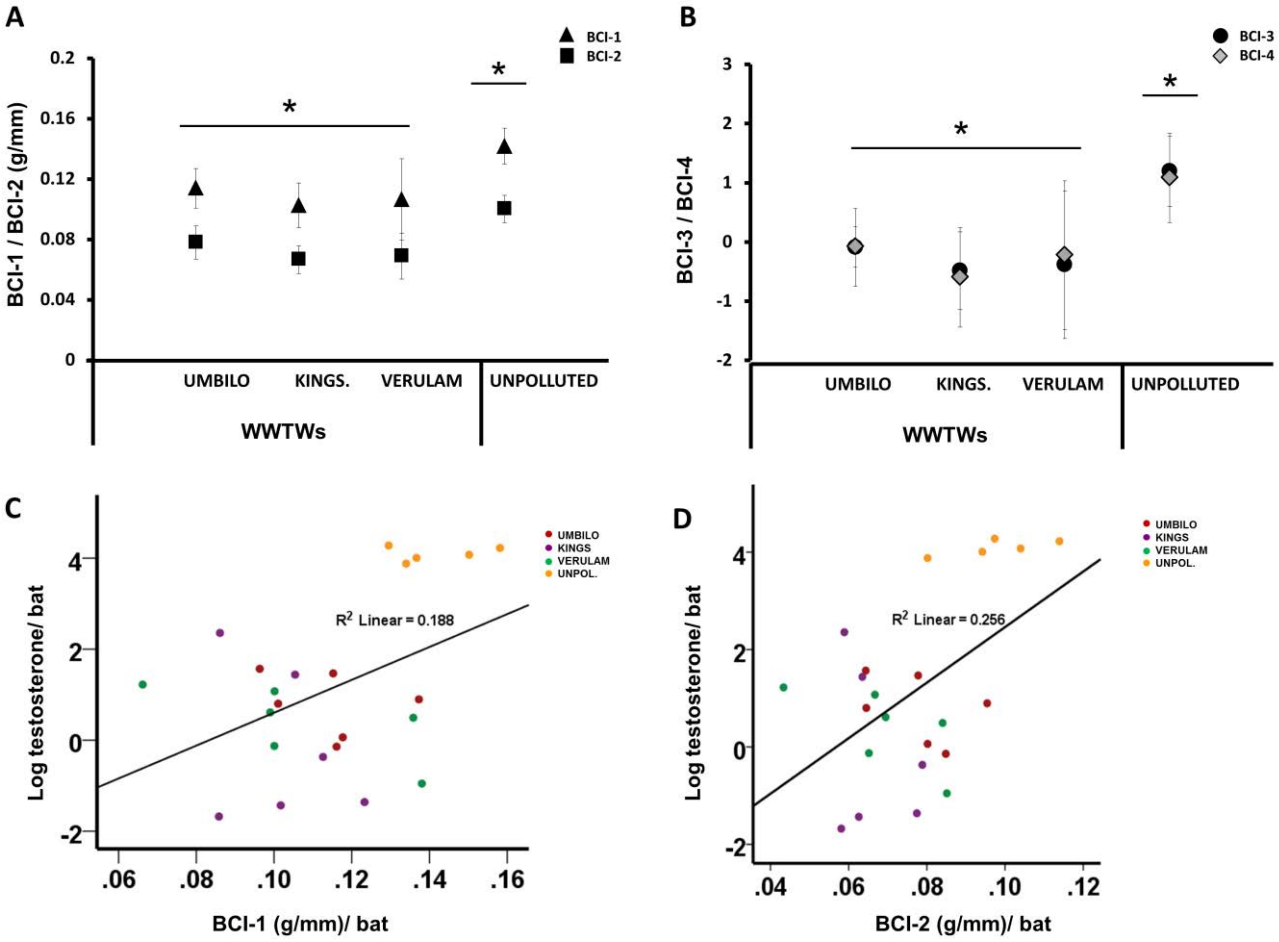

Fig. 4. Mean (A) BCI-1 and BCI-2, (B) BCI-3 and BCI-4, (C) relationship between BCI-1 and testosterone concentration and (D) relationship between BCI-2 and testosterone concentration of *N. nana* at the Umbilo, Kingsburgh and Verulam WWTWs and unpolluted sites at Umdoni Park. (* indicates significant differences between the sites at the $P < 0.05$ level). $N = 23$; (Mean ± Std. dev.)

5.5. Discussion

The aim of this study was to investigate the reproductive system of male *N. nana* foraging at WWTWs in the context of four hypotheses of EDC effect. In accordance with our predictions, we found significantly lower plasma testosterone concentrations, and lower body condition in male *N. nana* foraging at WWTWs than in males from the unpolluted reference sites at Umdoni Park. We did not, however, find support for our predictions that bats at WWTWs would have smaller bacula or lower GSI.

Testosterone is the main male sex hormone in vertebrates and is responsible for the development of the male internal reproductive ducts and external genitalia (Guillette, Jr., et al., 1996). It is also directly involved in the production of sexual signals such as displays showing off specific phenotypes that constitute reproductive effort (Mills et al., 2009). We found that testosterone concentration in bats at the WWTWs was notable, 25.89 ± 5.99 fold lower than bats from unpolluted sites. Although bat testosterone levels have a wide range of variability during different stages of the reproductive cycle (Hosken et al., 1998), we sampled bats from unpolluted sites at both the start and end of the sampling period, and overall testosterone levels were still consistently much higher than those of the WWTW bats.

In males, testosterone binds to androgen receptors with the highest affinity and also mediates the actions of the other androgens, androstenedione, dehydroepiandrosterone, and dehydroepiandrosterone sulfate (Kaufman and Vermeulen, 2005). The low testosterone in WWTW bats is likely due to the mechanistic action of EDCs which directly disrupt hormone signalling by binding to these androgen receptors (Pelch et al., 2010). An excess of estrogenics may also lower testosterone in exposed males by impacting the aromatase system which converts testosterone to estradiol (de Ronde and Jong, 2011). Serum testosterone levels were lowered by approximately 2-fold in laboratory controlled experimental exposures of male mice to EDCs (Wan et al., 2011). In male rhesus monkeys (*Macaca mulata*) exposed to PCBs, normal plasma testosterone levels were nearly halved, in conjunction with decreased testicular size and adversely affected spermatogenic activity (Ahmad et al., 2003).

The low testosterone concentrations in male *N. nana* at WWTWs may also be related to their copper and zinc levels. Chang et al. (2011) found that Korean men with a high level of Cu and elevated Cu/Zn ratio in hair tissue had decreased serum testosterone. Similarly, we previously found that *N. nana* at WWTWs had significantly higher kidney Cu and significantly lower liver Zn than bats from unpolluted sites (Naidoo et al., 2016). Thus, the high Cu/Zn ratio in WWTW bats may contribute to decreased synthesis of testosterone. Further, a high Cu/Zn ratio has shown to correlate with decreased sperm quality (Yuyan et al., 2008).

Testosterone directs spermatozoa development and sperm production (Garamszegi et al., 2005). Although we did not assess semen in the present study, the lower testosterone levels suggest that WWTW males may have lower sperm production and quality, and hence less efficient sperm competition (Parker, 1970). Male and female *N. nana* roost together and group membership is

labile, suggesting a promiscuous mating system and sperm competition (Bernard et al. 1997). Thus, males which have the genetic capacity to cope better with pollutant exposure, may dominate fertilization of resident females and thus have a higher reproductive output (Garamszegi et al., 2005). However, wives of human male WWTW workers that were exposed to WWTW-pollutants around the time of conception experienced high incidences of fetal loss (Morgan et al., 1984).

Because testosterone is linked not only to sexual differentiation and fertility, but also to sexual behaviours, *N. nana* at WWTWs may face negative impacts on mating success in addition to the physiological effects of EDC exposure (Blocker and Ophir, 2013). Demasculinization, ornament production, courtship, territoriality, male attractiveness to females, male-male competition and aggression, sexual receptivity, sexual arousal, vocalization, mounting females and copulatory behaviour in males can be altered in response to EDCs in a range of taxa (reviewed in Frye et al., 2012; Shenoy and Crowley, 2011). In addition, other socially relevant behaviour such as play (Hotchkiss et al., 2003), the motivation to explore and anxiety was reduced (Farabollini et al., 1999), and pain threshold increased in bisphenol-A treated male rats (Aloisi et al., 2002).

The low plasma testosterone levels that we found suggest recent pollutant exposure in these adult males. However, prenatal exposure to EDCs also programs adult gene expression, therefore the adult's ability to produce testosterone may also be impaired because of long-term developmental effects (Hotchkiss et al., 2002, Pelch et al., 2010). During pre-natal and early life, testosterone is a key androgen involved in the development of the baculum, or os-penis (Yonezawa et al., 2011). The distal part of the baculum develops as cartilage during embryogenesis, followed by the growth of the shaft and proximal portion, and is completed by ossification of the structure (Smirnov and Tsytsulina, 2003). During early postnatal life, the baculum continues to grow for approximately two months in vespertilionid bats (*Nyctalus noctula, Vespertilio murinus*) (Smirnov and Tsytsulina, 2003). Various mechanical and behavioural hypotheses have been proposed for baculum function in bats (Herdina et al., 2015). These include structural support, expansion of the female cervix for optimal sperm deposition (Long and Frank, 1968), coital locking (Dyck et al., 2004), and protracted copulatory time (Dixson, 1987). Contrary to our prediction, we did not find significant reductions in size of bacular parameters of bats from WWTWs. This suggests that testosterone activity and baculum development was not significantly disrupted during fetal and early life. By contrast, in East Greenland polar bears (*U. maritimus*) (Sonne et al., 2006), wild mink (*N. vison*) from Sweden (Persson and Magnusson, 2015), and river otters (*Lontra canadensis*) from the USA (Henny et al., 1996), EDC exposure decreased baculum size. In the case of *N. nana*, it may be that the pollutant exposure dose was not high enough to adversely affect baculum development. It may also be a

consequence of the mixture of EDCs that the bats are exposed to at WWTWs. Although primarily androgen-mediated, estrogen also plays a role in early baculum growth in rats (Yonezawa et al., 2011). Specific EDCs such as some metals (Iavicoli et al., 2009) and PCB congeners (Hamers et al., 2011) have estrogenic effects, and may therefore oppose disrupted testosterone by having a stimulating effect on baculum growth. Alternatively, it may be linked to the ecology of *N. nana*, where lactating bats and their juveniles form maternity roosting colonies (Bernard et al., 1997; LaVal and LaVal, 1977). Pregnant and lactating bats may change their foraging habits to sustain high-quality milk production (Kurta et al., 1989) by selecting prey items that are high in energy and calcium (Barclay and Harder, 2003; Dietz and Kalko, 2007; Kunz, 1974). It is thus possible that suckling male *N. nana* are not exposed to pollutants if their lactating mothers do not feed primarily on chironomids at WWTW tanks during the period which coincides with baculum development.

Although we found no changes in baculum morphometrics as an effect of early life exposure to EDCs, the disruptions to testosterone levels that we observed in adulthood may in turn affect other sex organs such as the testes (Garamszegi et al., 2005). However, contrary to our prediction, we did not find lower GSI in WWTW bats, indicating no whole organ effects on testes. Although findings of pollutant effects on testes are common, there are some cases where no significant change in testes size was noted. For instance, testis size was not associated with EDC levels in wild mink (*N. vison*) (Persson and Magnusson, 2015). In mink, testes size varies during the breeding cycle, even within short timeframes (Persson et al., 2011). Similarly, in bats there is some degree of variation with natural reproductive cycles (Hosken et al., 1998; Racey, 1974). Although we controlled for differences in time between sampling at each site (see Methods), this natural variation may have masked a possible effect of EDCs on testis size. In addition, intermittent or differing volumes of EDC release, and the opposing effects of different pollutants at WWTWs may prevent a directional effect on whole organ size. For instance, exposure to the EDC, Aroclor 1242 causes testes size to decrease (Ahmad et al., 2003). On the other hand, tissue lesions such as inflammation from metals may cause organ enlargement (Naidoo et al., 2016). We have previously found higher levels of the metals, Fe and Cu, along with significantly higher renalsomatic and hepatosomatic indices in *N. nana* at WWTWs than in bats from unpolluted sites (Naidoo et al., 2016). Thus, further histopathological analysis of the testis should be performed to reveal the nature of tissue/ spermatogenic damage within the testes to explain why GSI did not differ between sites.

Although we did not find an effect on the sex organs, ie. the baculum and testes, the decreased testosterone levels in *N. nana* at WWTWs may still ultimately affect the individual's ability to acquire mates and reproduce, and thus impact fitness, as discussed earlier. Body condition is an

important factor in determining an individual animal's evolutionary fitness (Green, 2001). The body condition index provides a measure of the energetic state of an individual, specifically the relative size of energy reserves such as fat and protein (Krebs and Singleton 1993). In many taxa, fitness parameters related to reproduction correlate strongly with body condition (Dobson, 1992). As predicted, all four measures of body condition (BCI-1 to BCI-4) were significantly lower in bats from the WWTWs than from the unpolluted sites, suggesting lower quality males. A low BCI indicates that more energy reserves are being utilized for survival than for reproductive effort (Jakob et al., 1996). Low BCIs are common in animals living in poor quality habitats (Schulte-Hostedde et al., 2005) or infected with disease (Dunlap and Mathies, 1993). Energy reserves are then channelled into maintaining basic bodily functions to survive. Similarly, in *N. nana* at WWTWs, we previously found physiological injury such as DNA damage that requires additional energy to repair, and increased erythrocyte production to counter oxidative stress from pollutants (Naidoo et al., 2015). Thus, *N. nana* at WWTWs direct their energy reserves into basic functioning and detoxification processes, resulting in a lower body condition. This channelling of energy away from reproductive processes provides an additional explanation for the low testosterone observed in WWTW bats, since maintaining high testosterone levels has been shown to be energetically expensive (Emerson and Hess, 1996). In support of this premise, the only significant correlation we found between variables quantified in the present study was between testosterone and BCI-1/BCI-2 where bats from the unpolluted sites had higher testosterone and higher body condition compared to bats from the WWTWs. High testosterone levels drive stronger secondary sexual signals and increase reproductive functioning, yet the hormone also imposes health risks (Alonso-Alvarez et al., 2007). For example, the immunocompetence handicap hypothesis proposes that high circulating levels of testosterone entails a cost because of the higher risk of mortality from immunosuppression (Folstad and Karter, 1992). Additionally, the oxidative hypothesis postulates that high testosterone generates oxidative stress in mammalian tissues (Alonso-Alvarez et al., 2007). When a sexually selected marker, in this case high testosterone, is present, the handicap it imposes may demonstrate that the genotype is of high quality (Zahavi, 1975). Bats from the unpolluted sites had high testosterone levels that positively correlated with their high BCIs, suggesting that they can afford the testosterone handicap, and still maintain body condition. In WWTW bats however, low testosterone and its associated low BCI, suggests that in addition to EDC effects on testosterone directly, these bats cannot afford the high testosterone handicap because survival costs are increased from counteracting pollutant exposure effects.

To conclude, our results show that male *N. nana* foraging at WWTWs suffer negative alterations to the endocrine system and body condition with impacts for evolutionary fitness. Testosterone levels

link male morphology, behaviour, and, ultimately, reproductive success. Furthermore, alterations to the hormonal balance may lead to diseases such as cancer, cryptorchidism and various types of skin diseases (Kumar et al., 2008). Future work should thus investigate long term physical damage to sperm, compositional structure of primary sex organs, behavioural changes and number of offspring produced by male *N. nana* at WWTWs. In addition, future studies should quantify EDCs at the particular WWTWs to understand which and how specific combinations of environmental pollutants affect reproduction. Finally, reproductive system alterations in female *N. nana* should be studied to understand the consequences for urban *N. nana* populations exploiting abundant food resources WWTWs.

5.6. References

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Chapter 6

Synthesis and conclusions

CHAPTER 6: Synthesis and conclusions

6.1. Synthesis/ Conclusions

River pollution negatively affects biodiversity (Azrina et al., 2006; Nedeau et al., 2003; Vörösmarty et al., 2010); it significantly lowers species richness and population abundances. Specifically, there are negative health effects in susceptible organisms (Lydeard et al., 2004; Oberholster et al., 2008), however, evidence for the impacts of pollution are often available for a limited number of traits. The results of this thesis revealed that wastewater pollution affects the foraging ecology and negatively impacts the physiology (haematology, DNA integrity, kidney function) and condition (BCI, organosomatic indices, testosterone levels) of urban *N. nana* populations in Durban, South Africa. The results suggest that *N. nana* may benefit from WWTWs in the short-term but there may be negative implications for this species and for other river biota exposed to wastewater pollution both in the short and long-term.

Some bat species, pertinently urban adapters, exhibit increased activity at wastewater-polluted sites along rivers (Kalcounis-Rueppell et al., 2007; Vaughan et al., 1996). Similarly, *N. nana* abundance and feeding activity were significantly higher at wastewater-polluted sites (tank and downstream) than at sites located upstream of tank sites and effluent discharge into rivers (Chapter 2). This was related to the increased abundance of chironomid midges captured at wastewater-polluted sites and in the diet of *N. nana* (Chapter 2). Although chironomid midges are able to tolerate polluted environments, metal pollutants accumulate in the body of this organism (Krantzberg and Stokes, 1990). Indeed, the concentrations of metals associated with wastewater pollution (Cr, Cu, Fe, Ni, Zn, Cd and Pb) were generally lower in the water at unpolluted upstream sites than at wastewater-polluted sites, with the highest concentrations occurring at WWTW tanks (Chapter 2). Because metal pollutants accumulate in midges, *N. nana* foraging on them at sites polluted with effluent from WWTWs have a high chance of being exposed to these metals.

In support, I found evidence that metal pollutants at WWTWs are transferred to *N. nana* (Chapter 2). Essential metals (Cu, Zn and Fe) were detected in all *N. nana* tissue samples, however the toxic metals cadmium, chromium and nickel were mostly present in tissue of bats at wastewater-polluted sites. In addition, there was a significant positive relationship between the concentrations of metals in the kidney tissue samples and in water samples. Notwithstanding the low sample size and detection limit of the instrument, the presence of more toxic metals in *N. nana* tissue samples

collected from bats foraging at polluted sites is particularly notable. Cd has a tendency to accumulate in target organs over time, and Ni and Cr may be transferred from adult to young through lactation (Streit and Nagel, 1993). Thus, my results show the accumulation of specific metals in the tissues of *N. nana* at sites polluted by WWTWs. These results, in combination with the increased foraging behaviour of *N. nana* along wastewater-polluted rivers and at WWTWs, suggests that sub-lethal physiological effects are likely present in these individuals.

As predicted, I found that pollutant exposure at WWTWs causes sub-lethal effects in three aspects of biological functioning in *N. nana*: haematological and genotoxic parameters (Chapter 3), the detoxification system (Chapter 4), and in the reproductive system (Chapter 5). Bats ingest pollutants at the WWTWs that, in turn, are absorbed into the blood from the intestinal tract (Andersen et al., 1994). I found that primary physiological responses such as DNA damage and haematological changes are elicited in *N. nana* at WWTWs (Chapter 3). WWTW bats had significantly higher DNA damage and significantly lower antioxidant capacity than bats from unpolluted sites. This suggests that in WWTW bats, there is potential inadequate repair to double stranded DNA breaks, and a diminished capacity to cope with the excess reactive oxidative species produced from pollutants such as metals. Although there was no increase in micronucleus frequency, bats at WWTWs had significantly higher haematocrits, possibly due to erythrocyte production in response to certain pollutants such as Fe. These responses to pollutant exposure, detected in the peripheral blood and muscle tissue are precursors for more harmful long-term damage. For instance, the high occurrence of double stranded DNA breaks in *N. nana* may disrupt cell-cycle regulation and cell functioning, and may induce cell death (van Gent et al., 2001). Persistent DNA damage may ultimately lead to the formation of tumours and cancers (Pastink et al., 2001).

As blood circulates through the body, it passes through the liver and kidney, where toxic substances and pollutants are filtered for excretion or storage (Fox, 1991). I did not find support for my prediction that the detoxification organs of WWTW bats should have higher levels of toxic non-essential metals in liver and kidney tissue. Instead, I found significant differences in essential metals, and in one mineral nutrient (K) between sites (Chapter 4). I found higher Fe and lower Zn in the liver, which may lead to liver deterioration from an increased oxidation of lipids, proteins, and DNA (Fraga and Oteiza, 2002). In the kidney, I found higher Cu and lower K levels in WWTW bats. High levels of Cu have been shown to inhibit Na reabsorption into cells (Grosell et al., 2002). Because Na re-absorption in the kidney occurs via Na^+/K^+-ATPase pumps (Vander et al., 1994), the increased Cu exposure at WWTWs may lower Na and thus, lower K levels in cells, ultimately contributing to sodium deficiency and potassium deficiency linked disorders in bats at WWTWs.

Innate physiological mechanisms such as metallothionein may offer protection against tissue damage, however I found no significant protein-fold increase in MT1E in WWTW bats (Chapter 4). Consistent with my prediction that chronic exposure to pollutants should impact the detoxification organs, I found a significantly greater extent of histopathological lesions and higher organ indices in the liver and kidney of WWTW bats than bats from unpolluted sites (Chapter 4). This suggests that bats at WWTWS may have impaired organ function and metabolic disturbance. It is clear that pollutant exposure at WWTWs places stress on cells, organs and physiological functions in the bodies of exposed bats.

To counteract these sub-lethal effects, additional energy must be directed towards survival. Energy use is divided between basic physiological functioning (survival) and in acquiring mates and producing offspring (reproduction) (Jakob et al., 1996). The body condition index (BCI) is a proxy for the energetic state of an individual, specifically the relative size of energy reserves such as fat and protein (Krebs and Singleton 1993). It is thus an important indicator of an individual animal's evolutionary fitness (Green, 2001). I found that the BCI of male *N. nana* at WWTWs was significantly lower than BCI of males at the unpolluted sites (Chapter 5). This indicates that more energy reserves are being utilized for survival than for reproductive effort, suggesting lower quality males (Jakob et al., 1996). Thus, *N. nana* at WWTWs direct their energy reserves into basic functioning such as repairing DNA damage and detoxifying pollutants, resulting in a lower body condition. Maintaining high testosterone levels is energetically expensive (Emerson and Hess, 1996), and indeed, I found strikingly lower testosterone levels in bats from WWTWs compared to bats from unpolluted sites. In addition to lower energy availability for testosterone production, the bats at WWTWs are exposed to a wide range of endocrine disrupting chemicals (EDCs) that directly alter hormone synthesis and regulation. Various organic pollutants, and some metals found in WWTW bats such as Cd (Chapter 2), Fe and Cu (Chapter 4) are potent EDCs. I thus predicted that male *N. nana* at WWTWs should have reduced baculum morphometric parameters indicative of early-life exposure to pollutants, and lower gonadosomatic indices (GSI) indicating whole organ effects on testes. However, I did not find support for these predictions (Chapter 5). Although I did not find significant effects on male sex organs, the decidedly low testosterone levels in WWTW bats is likely sufficient to negatively impact fitness, because testosterone levels link male morphology, behaviour, and, ultimately, reproductive success.

The abundant supply of pollution-tolerant chironomid prey at wastewater-polluted sites along rivers and within WWTWs, favour high foraging activity of urban *N. nana* throughout the year at these sites. However, the ingestion of metals and organic pollutants cause short-term sub lethal responses

in haematological and genotoxic parameters, including higher levels of DNA damage, lower antioxidant capacity and increased haematocrits. In addition, the accumulation of pollutants in the liver and kidney result in disruptions in the balance of essential metals and mineral nutrients, histopathological damage within the tissue and whole organ effects. Finally, the combination of EDC exposure and lower energy reserves available for reproductive activity is linked to significantly lower testosterone levels and lower body condition in male *N. nana* at WWTWs. Collectively, my results indicate that various aspects of *N. nana* health are compromised by foraging at wastewater-polluted sites, bearing negative short-term and long-term implications for urban *N. nana* populations.

6.2. Potential consequences for N. nana *populations*

Sub-lethal physiological effects ultimately translate to larger scale impacts on physiology. For instance, an accumulation of DNA damage may lead to the disruption of cellular activities, and induce mutations. Consequently, serious health problems such as the development of cancers and organ disease may originate from these sub-lethal effects, resulting in a decreased lifespan and increased mortality. The stability of a population is negatively impacted by an increase in death rate (Krebs, 2008). Furthermore, increased mortality is particularly important for slow reproducing, long-lived species such as bats (Fairbrother, 2001).

In addition to increased mortality from chronic health problems related to sub-lethal pollutant exposure, mortality rates are affected by a number of biotic factors including predation and parasitic/ infectious diseases. In fact, pollutant exposure has been specifically linked to stressors that regulate these biotic factors, and hence risk of early mortality (Fairbrother, 2001). For example, impaired immune system functioning from exposure to metals and other pollutants has been associated with outbreaks of parasitic/ infectious diseases (Boyd, 2010; Fairbrother, 2001). This "contaminant-pathogen synergy" has been, for example, linked to the phocine distemper epidemic that struck the seal population in the Wadden Sea in the early 1990s, which coincided with depressed immune response from polychlorinated biphenyl contamination (Ross et al., 1995). Although the immune receptors of bats are unique and offer them greater resistance to pathogens than immune receptors of other mammals (Escalera-Zamudio et al., 2015), pollutant exposure may increase the vulnerability of bats to parasitic/ infectious diseases. For instance, 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). Furthermore, pollutant

exposure in bats contributes to immunosuppression (Lilley et al., 2013, Pilosof et al. 2013), further increasing their susceptibility to infection by White-nose syndrome and other diseases. This may be particularly important for urban adapters, such as *N. nana*, that are consistently active at polluted sites.

Together with mortality rates, population stability is also regulated by birth rates and thus, reproductive success. Pollutant exposure at WWTWs may result in negative effects on reproductive success, as discussed in Chapter 5. Over time, pollutant exposure may affect the functionality of sex organs, sperm quality, sex-specific behaviours, male to female sex ratios and an array of sex-related parameters. In addition to the lower fitness of male *N. nana* at WWTWs, trans-generational epigenetic effects of pollutants may further reduce the reproductive success of individuals, resulting in fewer offspring produced in these populations (Bickham et al., 2000). Pollutant effects on the reproductive health of *N. nana* individuals may therefore potentially extend to the population level.

In Europe, populations of specific bat species are declining in response to urbanization and its associated features, such as decreased water quality (Jones et al., 2009). In addition to physiological and direct molecular impacts of pollutant exposure, stochastic mutation processes in small populations may accelerate loss of genetic diversity (Kimura, 1962). Ultimately, urban *N. nana* populations may decrease in genetic diversity and thus number of individuals. *N. nana's* flexibility as an urban adapter to exploit anthropogenic resources allows it to thrive in urbanized landscapes. However, this ability may in itself be cause for a possible future decline of urban *N. nana* populations.

6.3. Potential consequences for the local ecosystem

Within an ecosystem, there are both direct (physiological functioning) and indirect (modifications to the food web) effects that may arise as a result of exposure to pollutants (Fleeger et al., 2003). Therefore, although there may be direct effects at each trophic level, they are invariably intertwined because of their interaction with each other (Wooton, 1994). For instance, neuroendocrine disturbances from exposure to pollutants, may elicit behavioural changes that affect biotic interactions (Boyd, 2010). Metals in particular, have been shown to affect predator-prey interactions by modifying both prey response behaviour and predator capture ability (Boyd, 2010). In predatory fish, respiration rate and swimming performance to capture prey is impaired by exposure to metals (Atchison et al., 1987). Loss of co-ordination, in bats, for instance, would greatly impair their

hunting ability. In insects, metals may result in behaviours that increase susceptibility to predation (Mogren and Trumble, 2010). For instance, metal can induce phototaxis where insects such as mayflies (*Adenophlebia auriculata*), move to areas with a high risk of predation (Gerhardt and Palmer, 1998). This is especially important at WWTWs, given that they are well-lit. Metals may also reduce locomotive ability, resulting in decreased escape ability from predators (Mogren and Trumble, 2010). In addition, infochemical disruption by pollutants may prevent the organism from detecting approaching predators (Klaschka, 2008). Thus, the effects of pollutants may affect multiple levels within the local ecosystem, resulting in trophic cascades (Nakano et al., 1999).

In addition to indirect effects of pollutants on biotic interactions within the ecosystem, the negative impact on the health of *N. nana* may affect higher levels of biological organization. I have shown that foraging at wastewater polluted sites affects *N. nana* on a cellular, organ and organismal level (Chapter 3, 4, 5). These effects may further extend to the population level (as discussed above – Section 6.2), as well as community and ecosystem levels. Pollutants may alter community and ecosystem composition by causing a variation in the abundance of one species to affect abundances of other species (Wooton, 1994). Species that are more sensitive to pollution effects are more likely to be reduced or lost from a community (Clements et al., 2009). Ultimately, the resistance and resilience of communities to respond to pollutant exposure will determine whether local ecosystem function is diminished (Chapin et al., 1997).

With the majority of urban rivers becoming polluted, the resident biodiversity may be under serious threat (Vörösmarty et al., 2010). To preserve river biota in landscapes altered by anthropogenic pollution, it is important that the mechanisms of chemical-induced damage are understood at each trophic and biological level. A detailed analysis of responses within ecosystems is rarely attainable due to the range of variables that have to be taken into consideration (Linder and Joermann, 2001). By unravelling pollutant effects in higher predators such as *N. nana*, much insight into these processes has been acquired.

6.4. Future work

In Chapter 2, I showed that *N. nana* abundance and feeding activity was significantly higher at wastewater-polluted sites than at unpolluted sites, because of the increased prey abundance at these sites. One important caveat of this study was that metal concentrations could not be determined in chironomid midges, mainly due to the low abundance of the insects at upstream sites. In future

studies, control midges that are not exposed to pollution could be laboratory-bred to compare with those captured at polluted sites. This will contribute to better quantification of metal bioconcentration (*BCF*) and bioaccumulation factors (*BAF*) (Linder and Joermann, 2001). In addition, future work should aim to quantify organic pollutants and the full range of metals in the wastewater mixture and in the prey that the bats ingest.

I have shown that sub-lethal damage from pollutants occurs in various aspects of *N. nana* physiology (Chapter 3, 4, 5). However, to establish that there is indeed damage within organs and tissue, individuals were sacrificed. Future work should aim to use non-destructive methods for investigating related physiological effects. In addition, this would enable a greater sample size and alternative statistical analyses. Future studies should also investigate longer-term health effects of the sub-lethal responses I quantified. For instance, developmental defects and the occurrence of specific diseases related to the metals found in high concentrations in *N. nana* tissue should be investigated. In addition to longer-term health effects, adjacent parameters to the responses I found should also be studied. For instance, I quantified a single hormone, testosterone. The significant effect of pollutant exposure on testosterone suggests disruptions to the endocrine system, where related hormones such as corticosterone may be affected. Finally, having found that wastewater pollution indeed induces harmful effects in these top predators, research in related fields such as chemistry, wastewater management and engineering should investigate possible solutions to increasing removal efficiencies of specific pollutants and preventing the direct exposure of urban wildlife to wastewater during the treatment process.

To conclude, the results of this study establish a link between exposure to wastewater pollution, foraging behaviour patterns, and sub-lethal alterations to haematological/ genotoxic parameters, detoxification organs and the reproductive system of *N. nana.* My results show that WWTWs, aimed to remove pollutants from the environment, can themselves act as a source of contamination and pose a threat to animals exploiting these habitats. Further research into the effects of WWTWs and polluted rivers, on urban biodiversity is vital, particularly because of the rapidly increasing rate of urbanization. WWTWs are an essential component of cities and may thus contribute to local and landscape effects of urbanization. In addition, pollutants in rivers pose an even greater threat to the resident fauna in the light of global warming (Clements et al., 2008). With increased water evaporation from rivers, it is estimated that the toxicity of metals and other pollutants will increase significantly due to lower dilution (Clements et al., 2008). Thus, the future functioning of urban river ecosystems faces serious threat unless the mechanisms of pollutant transfer and effects are elucidated.

6.5. References

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