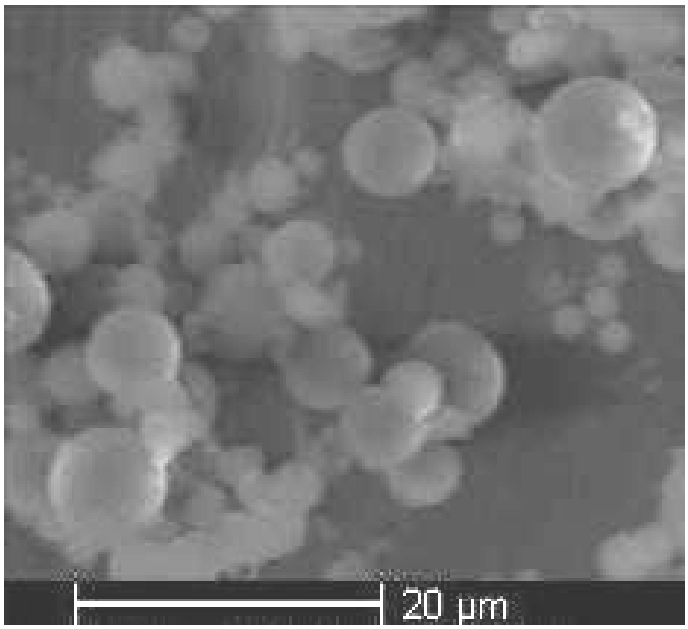
Avian urate spheres: A non-invasive method to biomonitor environmental pollution and stress in birds



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ABSTRACT

Birds are commonly used as biomonitors of environmental pollution, with most tests involving invasive or destructive sampling techniques. The need to develop and validate non-invasive techniques has long been recognised. From blood, eggs, feathers or guano, the last shows most promise in this field. However it constitutes both faecal and urinary excretions. The faecal component has serious analytical drawbacks from digestive processes and being comprised of both bio-available and unabsorbed components. In contrast the typically white urine part of guano represents substances emanating entirely from within the bird. Despite the analysis of urine (urinalysis) being widely and successfully used in mammals, its limited application to date in birds is at best misguided because it disregards the nature of avian urine. This thesis endeavours to show how the analysis of the (normally discarded) solid component of avian urine may provide a quantifiable measure of both environmental pollutant exposure and endogenous stress hormone concentrations in birds. The literature is reviewed with regard to birds as biomonitors of the environment and the use of non invasive sampling techniques, especially excreta collected from wild animals including birds. Avian renal physiology and urine composition is described with specific reference to current avian urinalysis methods and how these compare with the proposed use of avian urate spheres (AUS) for biomonitoring. It is also shown how the biomineralisation process of AUS formation is relevant to their collection, extraction and chemical analysis from bird guano. To investigate if AUS contents could be used as a measure of a bird's environmental pollution exposure, concentrations of lead, copper and zinc, were determined in urate spheres from domestic chickens (*Gallus domesticus*) exposed to a soil contaminated by these metals. Furthermore an attempt was made to compare metal concentrations in AUS with eggs, feathers and whole guano from the same birds. The results suggested AUS contained higher levels of the contaminating metals in exposed birds compared to control birds. However the aim to show the utility of AUS for biomonitoring the birds' metal exposure was not achieved because of experimental design limitations. A similar investigation was carried out into the suspected exposure of nestling seabirds to elevated metal concentrations in their fish diet. Metal concentrations in urate spheres from the seabirds were measured along with those in various body tissues of their young. This metal analysis, although limited by small sample size, provided no evidence of an elevated exposure when compared with values reported in the literature. Subsequent reanalysis of earlier tested fish samples showed normal metal concentrations, suggesting the earlier reported fish data had been incorrect. To determine if AUS can be used to measure biologically relevant levels of the avian stress hormone corticosterone, a series of experiments is described using captive great tits (*Parus major*). These involved the ELISA detection of excreted corticosterone in AUS extracts. The suppressive response to dexamethasone administration was measurable in AUS from these birds, suggesting a physiological validation. However many issues have still to be resolved concerning this method of measuring corticosterone levels in birds. The overall finding of this thesis is that the analysis of AUS may have potential value as a noninvasive sampling method to biomonitor environmental pollution and stress in birds.

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Chapter 1.

Introduction

Birds through the ages have been employed as biomonitors of our environment in many and diverse ways. Widely recognised examples are the first swallow heralding the UK summer, gulls locating shoals of fish out at sea and canaries detecting gas down a mine. Currently avian biomonitoring can employ a wide range of sampling techniques (Sutherland et al., 2004), with it being preferable to replace destructive, by non-destructive or non-invasive methods (Fossi et al., 1999). A commonly used non-invasive technique involves the analysis of excreta (Chame, 2003). The literature reports that concentrations of environmental toxins or their biomarkers in avian excreta can be used to quantify local pollution (Fitzner, 1995; Dauwe et al., 2000; Fossi et al., 1996), while excreted hormone metabolites can determine a bird's endocrine status (Goymann et al., 2002). This thesis explores the utility of the urine fraction of avian guano as a non-invasive sampling method to biomonitor environmental pollution and stress.

1.1. The importance of avian biomonitoring

Biomonitoring entails measuring changes in biological systems in response to perturbations of their environment. These upsets can be wide ranging and may be physical in nature such as habitat destruction or chemical from specific pollutants (Walker et al., 2001). An important characteristic of a biomonitor is that it only detects bioavailable changes, which impact on the biological system being monitored. The value of biomonitoring is highlighted in the case of interpreting pollutant concentrations in the environment; such concentrations tell us nothing about actual biological harm without knowledge of the pollutant's bioavailability (Ruby, 2004). The bioavailability of a pollutant can be complex, depending on many factors including its interaction with other substances in the environment and its propensity for absorption, metabolism and excretion by an organism (Ruby, 2004). As a result of these complications only biomonitoring can provide information on a pollutant's bioavailability and so its actual biological harm.

As birds are highly visible, wide ranging and ubiquitous higher animals, they represent ideal sentinels for monitoring environmental pollution and degradation (Hollamby et al., 2006).

1.2. The utility of avian urinalysis

The analysis of urine (urinalysis) for monitoring bodily functions and exposures has wide applications in man and other mammals (Doxey, 1983). The end product of nitrogen metabolism in birds is predominantly uric acid, which being sparingly soluble dictates that solid urate spheres are excreted by the avian urinary system (Braun, 2009). Approximately 5% of each urate sphere by dry mass is serum albumin derived from the bird's blood stream (Casotti and Braun, 2004), where this protein is the major carrier of blood borne substances (Peters, 1996). The main constituent of these urate spheres is uric acid, which across diverse bird species consistently makes up 65% of the spheres' dry mass (Casotti and Braun, 2004). This constant value allows for the hypothesis that the various sequestered substances in samples of urate spheres can be quantified against uric acid content. In this respect the analysis of avian urate spheres represents an ideal urinalysis technique to measure blood borne substances without the need to take blood samples.

1.3. Thesis aims

The overall aim of this thesis is to test the hypothesis that measurement of environmental contaminants and stress hormones in the solid component of avian urine, principally composed of urate spheres, is a valid non-invasive sampling technique when using birds to biomonitor environmental contamination and stress.

The objectives of this thesis are broadly to:

1) Develop a method of separating urine from faeces in bird guano;

2) Quantify the concentrations of chemicals in the extracted urine samples against uric acid content to allow sample comparison;

3) Devise and carry out experiments to measure a selection of suitable substances excreted in bird urine in order to validate this method of avian urinalysis.

1.4. Thesis outline

The thesis may be broadly divided into four sections, reflecting my research into avian urinalysis. The first section (Chapter 2) reviews the literature on birds as biomonitors of the environment and how avian renal physiology provides the rationale for using the solid component of bird urine as a non-invasive biomonitoring technique.

The second section (Chapters 3 and 4) reports on studies using avian urate spheres to determine metal exposure in birds and compares this new technique with currently used destructive and non-destructive methods. Chapter 3 reports on a project to measure lead (Pb), copper (Cu) and zinc (Zn) concentrations, in extracted urine from domestic chickens kept on soil contaminated by these heavy metals and compares them with concentrations measured in eggs, feathers and guano from the same birds. As a control, similar samples were analysed from unexposed birds. Chapter 4 is an account of an investigation into the suspected heavy metal contaminated fish diet of nesting seabirds on the Farne Islands off the coast of Northumberland in the UK. This chapter compares metal concentrations in urate spheres from guano deposits with various body tissues from dead nestlings and their fish diet.

The third section (Chapter 5) describes the development of a method to measure levels of the stress hormone corticosterone in birds by the analysis of their urate spheres. This involved the experimental manipulation of the stress hormone (corticosterone) in captive great tits (*Parus major*).

The final section (Chapter 6) draws conclusions from this research and discusses how future studies may resolve issues relating to the validation of this proposed method for avian urinalysis.

1.5. Conclusions

Birds are recognised sentinels of environmental change but to date avian biomonitoring predominantly involves detecting population changes (Peakall, 2000) or pathology in birds found dead (CEH, 2003/04). Consequently these changes record post-impact effects that once identified may be difficult to reverse, which is a corollary to the current predicament over measuring climate change (Solomon et al., 2009). A better approach would be to repeatedly monitor pollutant concentrations in living birds enabling action to be taken before lethal concentrations are reached. The proposed technique of avian urinalysis presents a widely available non-invasive sampling method in birds, which could enhance their use as biomonitors and allow pre-emptive assessment of environmental changes.

Chapter 2.

A review of the literature relevant to using bird urine as a new biomonitoring technique

2.1. Introduction

This chapter reviews the role of birds as biomonitors of the environment and the use of non invasive sampling techniques, more especially using excreta from wild animals. Avian renal physiology and urine composition are described, with specific reference to comparing current avian urinalysis methods with the proposed use of avian urate spheres (AUS). The process of AUS formation by biomineralisation is explained and its relevance to the collection, extraction and chemical analysis of urate spheres from bird guano.

2.2. Birds as biomonitors of the environment

A typical definition of a biomonitor is an organism that is sensitive to, and shows measurable responses to, changes in the environment such as changes in pollution concentrations (US EPA).

Man has observed birds for a long time and from them has gained valuable insights into the environment. A classic example is fishermen at sea still to this day use flocking birds to locate shoals of fish, even in this high tech age of sonar detection. Birds being so visible also elicit concern in the general public when they become absent from increased mortality or failure to return in the case of migratory species (Bird Life International, 2008). The sudden decline in raptor populations in the 1960s alerted the world to the unforeseen consequences of widespread pesticide use, notably dichloro-diphenyl-trichloroethane (DDT) (Walker et al., 2001). More recently a similar population decline of vultures in South Asia has occurred from the use of the drug diclofenac in farm stock (Green et al., 2006; Swan et al., 2006). Such occurrences, it could be argued, make a lasting impression on public awareness of environmental toxicology issues. As a consequence many bird monitoring groups similar to those in the UK exist around the world, which study different aspects of avian population dynamics (Peakall, 2000). Such studies alert us to changes in bird population abundance and distribution, categorising different species in terms of their vulnerability, with those on the World Conservation Union (IUCN) Red List, being most at risk of extinction

(Butchart et al., 2004). The decline of birds such as the grey partridge (*Perdix perdix*) and sky lark (*Alauda arvensis*) in Britain is blamed on modern farming practices and as a result of public and political pressure has led to schemes rewarding farmers for using more environmentally friendly production methods (Peakall, 2000).

However bird monitoring restricted just to population studies only measures impacts which have occurred, by which point it may be too late to reverse the damage. For this reason a more pre-emptive approach is called for, where bird sampling will alert us to developing problems such as rising body concentrations of pesticides, in advance of them reaching fatal concentrations. An example of this is the Predatory Bird Monitoring Scheme (PBMS) run by the Centre for Ecology and Hydrology (CEH) in the UK (CEH, 2003/04). The PBMS is a long-term monitoring programme, set up in the 1960s, to measure concentrations of certain pollutants in the livers of discovered carcasses and in un-hatched addled eggs of selected predatory bird species. These pollutants include organochloride pesticides, polychlorinated biphenyls, mercury and anticoagulant rodenticides. The programme's rationale is that predatory bird species are more prone to poisoning due to bioaccumulation, as they are positioned at the top of food chains (Walker et al., 2001). The PBMS is however limited to studying only a few samples found by chance. For example in 2003 the total number of carcases submitted for analysis consisted of only 68 barn owls, 43 sparrow hawks and 39 kestrels (CEH, 2003/04), which constituted only a very small fraction of the total UK raptor population. In this respect there is a clear need for a more wide ranging sampling technique, to enable the measurement of pollutant concentrations in a wider proportion of such bird populations.

2.3. Sampling techniques for biomonitoring

From the many sampling techniques employed to use avian species as biomonitors (Hollamby et al., 2006), the non-destructive methods hold the most promise (Fossi, 1994). Destructive monitoring methods from culling and post-mortem sampling are finite and although unsavoury may be more acceptable to the public if very common or pest species are used (Hollamby et al., 2006). An example is the use of house sparrows (*Passer domesticus*) (Swaileh and Sansur, 2006) or feral pigeons (*Columba livia*) (Nam et al., 2004b; Loranger *et al.*, 1994), to monitor urban metal pollution. However destructive methods in dwindling populations, especially for the purpose of enquiring why they are in decline, would clearly be questionable. Even when a sampling method is defined as non destructive such as blood collection, it may constitute invasive and

stressful interference to the animal (Kurien et al., 2004). This could for example scare a bird from its nesting site or cause other unforeseen consequences leading to its further decline. In this respect non invasive or non disturbing methods are preferable for endangered species (Fossi et al., 1999); having no measurable effect on the animal or on the parameters being studied such as stress hormones (Goymann et al., 2002). These non-disturbing methods sample materials remaining after the animal has vacated a site, and may include: excreta, hair, feathers or un-hatched eggs (Sutherland et al., 2004). Each of these methods has its limitations and one method may be more suited than another to measure the parameter being monitored. Excreta may be dispersed or contaminated after being passed; hair and feathers may only be shed seasonally as during a moult and eggs will only monitor female birds in the breeding season. The uptake, assimilation in tissues and excretion of environmental chemicals to which birds are exposed can vary respectively with bioavailability (Ruby, 2004), tissue type (Nam et al., 2005) and physiological status (Finley and Dieter, 1978). Of the non-disturbing sample materials collected the most widely used is excreta, more especially faeces.

2.4. A brief overview of faecal and urine sampling techniques

In human and veterinary medicine faeces and urine have a wide range of applications for clinical diagnostic tests, described respectively as coprological and urological sampling techniques (Doxey, 1983). Ingested poisons, disorders of the digestive system caused by bacterial, viral and parasitic infections and organ disorders such as pancreatic insufficiency and liver damage can all be detected by monitoring of faecal samples (Doxey, 1983). Urine, likewise, is used to detect disorders specific to the urinary system such as urolithiasis (stones), bladder infections, tumours and several types of kidney disease (Doxey, 1983). Urine analysis has a wider range of applications than faecal analysis because it can reflect concentrations of many blood constituents, so measuring conditions throughout the whole body. Some examples are sex hormones for reproductive status (pregnancy tests); metabolites for metabolic disorders (ketosis and diabetes) and blood borne toxins or pharmaceuticals (Doxey, 1983). Added to this, urinary proteomics is emerging as a powerful non-invasive tool for the diagnosis and monitoring of many human diseases (Hanash, 2003), including coronary artery disease (Zimmerli et al., 2008).

For human biomonitoring (HBM), blood and urine are by far the most approved matrices to measure human exposure to chemical substances (Angerer et al., 2007).

Consequently, I propose urine rather than faeces represents a better matrix for biomonitoring in birds.

This is based on there being more diagnostic applications for using urine compared with faeces, combined with the complications of diet and digestive processes on faecal samples (Klasing, 2005) and their complex extraction protocols (Palme, 2005).

In the field of experiments on domestic and laboratory animals, metabolism cages are widely used to collect faecal and urine samples (Kurien et al., 2004; Wasser et al., 2000). Typically the purpose is to measure food digestibility or excreted metabolites or chemicals such as hormones and drugs. However the complication of faeces and urine co-mingling commonly occurs in female test individuals unless catheterisation is employed (Kurien et al., 2004). Such a problem is analogous to birds that pass faeces and urine as a single entity called guano from their cloaca, which makes separate analysis difficult (Palme, 2005).

2.5. Sampling of wild animal excreta

In free living wild animals unlike under domestic or laboratory conditions (Kurien et al., 2004), the collection of urine is impractical without capturing and usually catheterization of individual animals. For this reason urine sampling in such cases is rarely reported in the literature, with the exception of snow urine. Snow urine collection from wolves (Hausknecht et al., 2007; Valiere and Taberlet, 2000), elk (Pils et al., 1999; DelGiudice et al., 1991) and seals (Constable et al., 2006) is possible when urine freezes after being passed in subzero temperatures and so preserved for later collection. Such a method clearly has limited applications for most wild animals.

Faecal samples however are easily collected after an animal vacates a site, avoiding observer interference. An added advantage of faeces is that the gross morphology is often species-specific (Chame, 2003). The easy identification and ready availability of faeces may explain the many reported applications for faecal analysis in non-invasive sampling studies of wild animals, such as sex and stress hormone analysis (ANYAS, 2005; Dehnhard et al., 2001; Foley et al., 2001), pollution derived liver damage in birds (Fossi et al., 1996) and heavy metal ingestion (Dauwe et al., 2000; Pokorny, 2004; Fitzner, 1995).

However when quantitative measurements of faecal constituents are required such as the concentrations of excreted stress hormones (Mostl et al., 2005); faecal composition can have a marked effect on concentrations even within the same individual (Klasing, 2005), giving inconsistent results (Goymann et al., 2006). This problem arises because

there is no single constant parameter in faeces, against which constituent concentrations can be measured. In contrast, in urine samples, creatinine is used to compensate for fluctuations in composition i.e. dilution (Pils et al., 1999). Measuring faecal constituents against dry matter is reported to resolve this problem (Wasser et al., 2000). However this only removes the complication of faecal water content and does not compensate for variations in food digestibility, which can change with diet and transit time (Klasing, 2005).

Taking these factors into consideration it would be most advantageous if a method of non-invasive urine sampling could be devised for free-living animals, which does not require capture or direct interference. I hypothesise this requirement may be feasible in respect to free living birds. The grounds for this hypothesis stems from birds being uricotelic, excreting mainly uric acid as the end product of nitrogen metabolism.

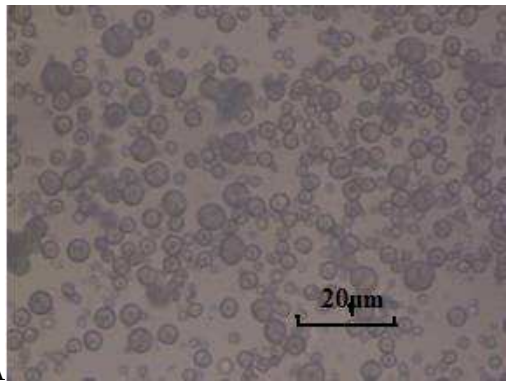
As uric acid is practically insoluble (McNabb and McNabb, 1980), vast amounts of body water would be wasted if it was to be passed as a solution in avian urine. To overcome this problem a mechanism of biomineralisation (Mann, 2001) packages the urinary excreted uric acid with protein as minute spheres (Janes and Braun, 1997), which birds pass as a white paste-like suspension (Tschopp et al., 2007). As the solid component of avian urine, these avian urate spheres (AUS), can be collected noninvasively after a bird has vacated a site.

2.6. Bird urine composition and physiology

2.6.1. Urine composition

Bird urine is typically white with a paste-like consistency (Tschopp et al., 2007); however most authors define avian urine as the liquid supernatant following centrifugal separation from the solid urate fraction (Styles and Phalen, 1998). This may be because the supernatant provides a urine sample which appears similar in nature to mammalian urine. Furthermore this liquid component can also be analysed in a comparable manner for specific gravity and chemical constituents (Styles and Phalen, 1998). However this fluid is only a small fraction of the true urine output of the avian kidney because the majority of urinary excreted solutes reside in the AUS (Casotti and Braun, 2004). AUS (Fig. 2.1A & B) are an example of biomineralised spherulitic structures (Taylor and Simkiss, 1989). The apparent fold in the centre of each sphere seen in the light microscope view (Fig. 2.1A) is a product of light interference (Folk, 1969; Canti, 1998)

and so not seen in the electron micrograph (Fig. 2.1B). Typically AUS are composed of 65% uric acid combined with 5% serum proteins, with added inorganic ions mainly of potassium, sodium and calcium (Casotti and Braun, 2004). Although the process of urate sphere formation is common to all uricotelic organisms, which along with birds include reptiles, molluscs and insects, the exact biochemical mechanism of their synthesis is still unknown (Casotti and Braun, 2004).

A 

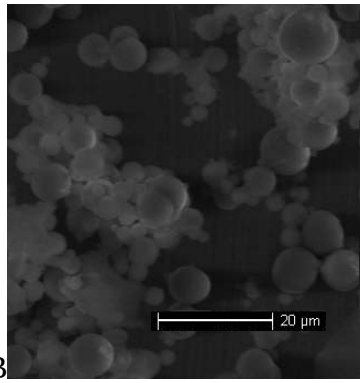
B 

Figure 2.1. A: Medium power light microscope view and B: Scanning electron micrograph of urate spheres from a domestic chicken (*Gallus domesticus*).

2.6.2. Urine formation

It is believed the purpose of urate sphere formation is an evolutionary solution to packaging the poorly soluble uric acid for excretion. Without this mechanism, uric acid would, under the normal process of crystallisation, precipitate as larger plate-like crystals and inevitably block the renal tubule (Janes and Braun, 1997).

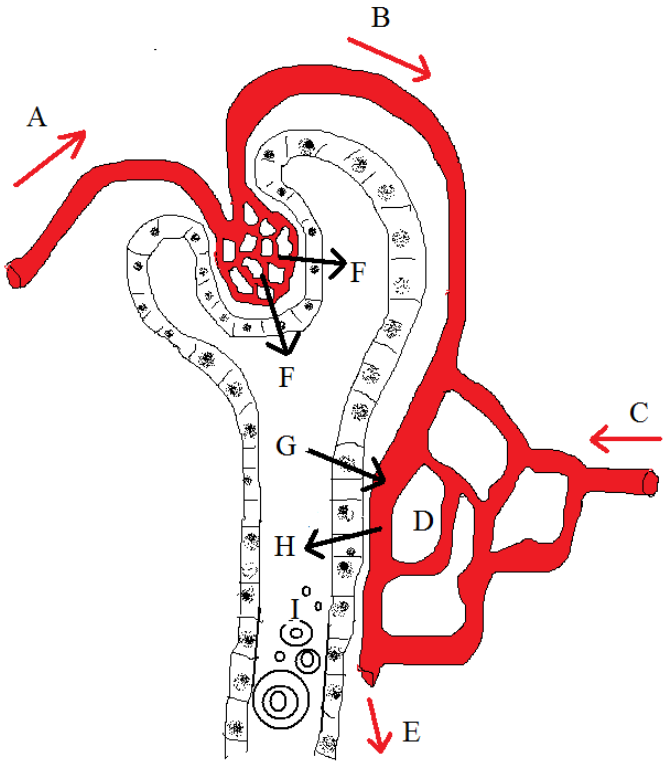


Figure 2.2. A simplified diagram of the avian upper nephron showing the blood supply and site of urate sphere formation. A: Afferent arteriole B: Efferent arteriole C: Afferent portal vein D: Peritubular sinus E: Efferent portal vein F: Glomerular filtration G: Tubular reabsorption H: Tubular secretion I: Urate sphere formation in proximal tubule lumen.

This process of solute precipitation in the form of urate spheres (Fig. 2.2.) significantly contributes to the avian proximal tubule achieving the reported 95% re-absorption of filtered water (Goldstein and Skadhauge, 2000). This is because the formation of urate spheres takes out of solution many osmotically active solutes such as uric acid, albumin and inorganic ions (Janes and Braun, 1997). The reduced osmotic potential of the filtrate makes it easier for water re-absorption, a process linked to active sodium uptake by the tubule cells (Brokl et al., 1994). After leaving the proximal tubule the liquid fraction of the filtrate may be further concentrated, in the case of mammalian-type nephrons, which have loops of Henle (Goldstein and Skadhauge, 2000). However no further modification to the urate spheres occurs following their formation in the lumen of the proximal tubule (Casotti and Braun, 2004).

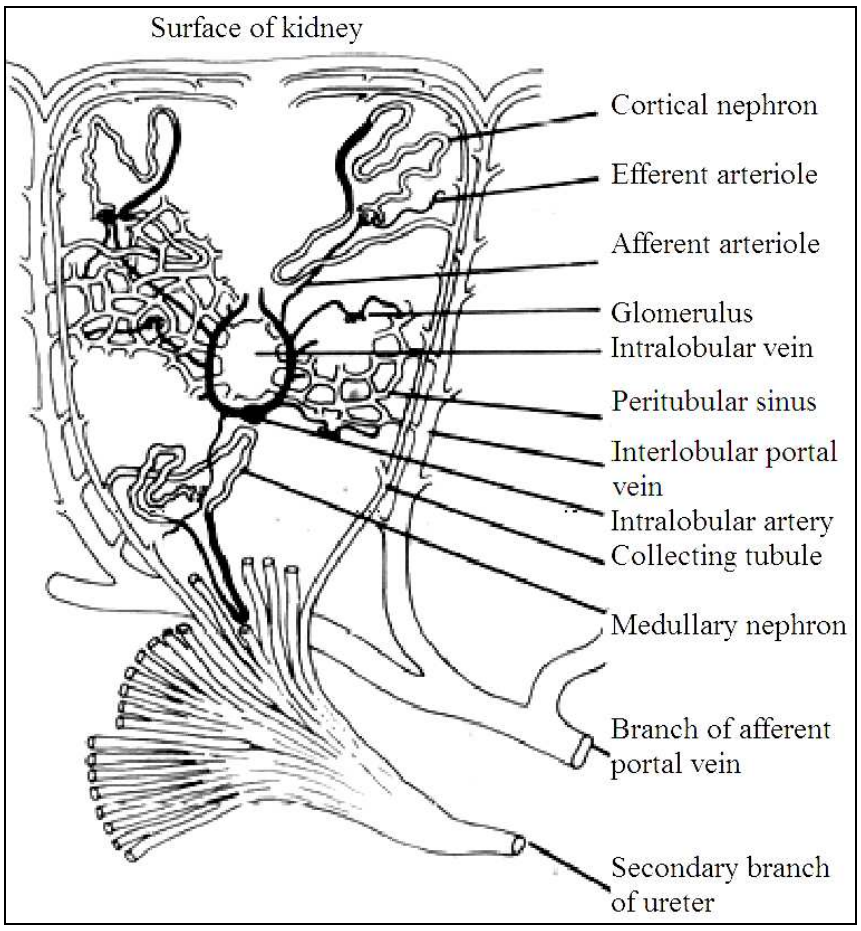


Figure 2.3. Diagram of a histological section of a renal lobule from a domestic fowl showing the location and morphology of the two nephron types: medullary (mammalian) and cortical (reptilian). The black part of these nephrons (intermediate segment) only forms a loop of Henle in the medullary nephron (from King and McLelland, 1975).

In birds arginine vasotocin (AVT) is equivalent to the anti-diuretic hormone (ADH) of mammals, which reduces an animal's water loss by concentrating its urine. This is achieved by a combination of reducing the glomerular filtrate rate (GFR) through vasoconstriction, and increasing water reabsorption by enhancing tubular permeability (Goldstein, 2006). It is widely stated in the literature that compared to mammals, birds have a limited ability to concentrate urine above that of plasma (Braun, 2003). The explanation for this being that many birds have a high percentage of reptilian (cortical) nephrons (Fig. 2.3), which lack the loop of Henle present in mammalian (medullary) nephrons, essential for urine concentration by AVT (Dantzler, 2003). Hummingbirds,being an extreme example, have >99% of such reptilian type nephrons (Casotti et al., 1998), making them totally unable to produce urine hyperosmotic to plasma (Lotz and Martínez del Rio, 2004). However this only relates to the residual

fluid fraction of avian urine leaving the nephron and ignores the enormous water-preserving benefit of urate sphere formation (Goldstein and Skadhauge, 2000).

2.6.3. Post renal modification of ureteral urine

To compensate for possessing reptilian type nephrons, birds have a strategy to retrieve water (and sodium) from their urine by a process of post-renal modification (Laverty and Skadhauge, 2008). This involves the reflux of some ureteral urine into the avian lower bowel, constituting the coprodeum, colon and caecal sacs (Fig. 2.4.). For example in the emu (*Dromaius novaehollandiae*), which has almost exclusively reptilian type nephrons, water is reabsorbed from urine refluxed into the lower bowel. While in the opposite and unique case of the ostrich (*Struthio camilus*), with predominantly mammalian type nephrons, no post renal water reabsorption takes place (Laverty and Skadhauge, 2008). This is in part a consequence of the ostrich having a functional urinary bladder (Fig. 2.4B), which precludes any reflux of urine into the lower bowel (Duke et al., 1995).

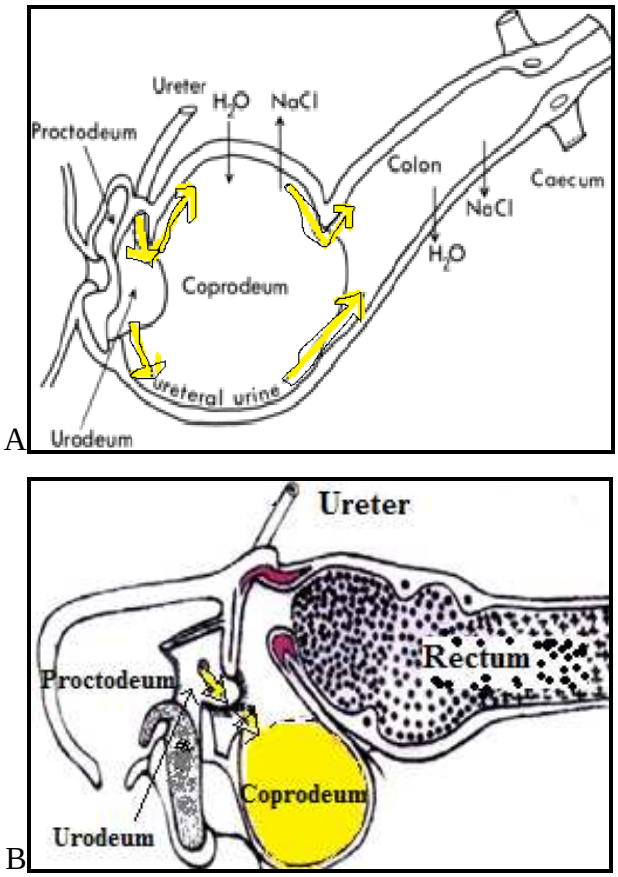


Figure 2.4. Sagittal sections through the lower bowel and cloaca of A: domestic fowl (*Gallus domesticus*) and B: ostrich (*Struthio camilus*), yellow signifies urine, showing respectively the reptilian and mammalian adaptations to post renal handling of urine in birds (after Laverty and Skadhauge, 2008).

As a result of urine reflux, some urine becomes intimately mixed with faecal excreta in birds prior to its evacuation as guano. In the case of Gambel's quail (*Callipepla gambelii*), over 60% of the excreted urate spheres are degraded in the process of urine reflux (Braun, 2009 Fig. 2.5). The relevance of urine reflux to avian urine analysis is that a substantial quantity of urine in the form of urate spheres may not be available for analysis. Furthermore the faecal component of guano will represent a mixture of excreted metabolites originating from both the renal and digestive systems. To illustrate this point several studies measuring excreted hormone metabolites in bird guano describe them as exclusively faecal concentrations (ANYAS, 2005). However as birds have been shown to have similar excretion routes to mammals for such metabolites, guano concentrations include substantial amounts of urinary excreted hormones (Lepschy et al., 2008).

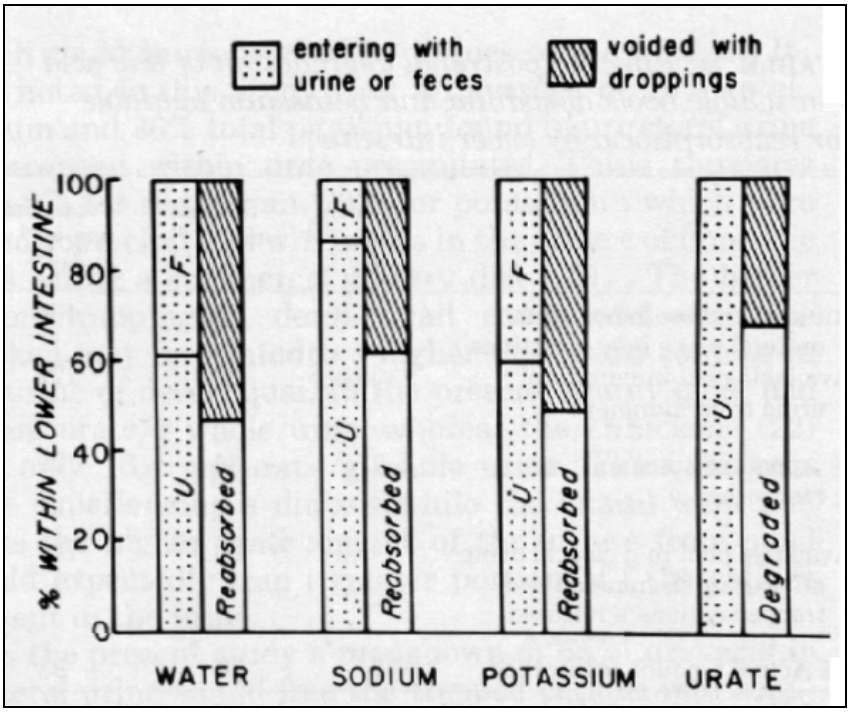


Figure 2.5. Graph showing the modification of urine and faecal constituents in the lower digestive tract of Gambel's quail (*Callipepla gambelii*) (from Braun, 2009).

2.6.4. The avian renal portal system

Anatomically a portal system (or circulation) exists when blood leaving an organ does not return directly to the heart but instead enters another organ first (Sisson and

Grossman, 1940). The most commonly cited example of this is the hepatic portal system, where blood leaving the intestine enters the liver first before proceeding to the heart. The purpose of the hepatic portal system is that intestinally absorbed substances, such as nutrients, go first to the liver for processing prior to distribution throughout the body via the general circulation. However a side effect of this is that some absorbed substances are bile excreted and then reabsorbed. This cyclic process is called enterohepatic recirculation (Roberts et al., 2002). The significance of enterohepatic recirculation is that it can prolong the period of time chemical substances remain in the body (Roberts et al., 2002).

In birds a renal portal system exists which incorporates several blood vessels draining the hind end of the bird. These include the caudal mesenteric vein from the cloaca, colon and caecal sacs, also the ischiatic and external iliac veins from the hind limbs (King and McLelland, 1975). The significance of the caudal mesenteric vein flowing into the (caudal) renal portal vein is that it supplies the kidney with blood carrying substances absorbed from the lower bowel. This afferent portal vein blood joins that of the efferent glomerular arterioles (see Fig. 2.3.) to bathe the tubular structures of the nephron, where the tubular excretion phase of urine formation takes place (King and McLelland, 1975). A clinical consequence of the avian renal portal system, is that a drug given by injection into a bird's leg, can result in its direct urinary excretion and so prevent its therapeutic action (Coles, 2007).

The combination of birds having a renal portal system and the process of urine reflux into the lower bowel may result in substances being repeatedly recycled through the kidneys. This has important implications for avian urinary analysis, because it may prolong the presence of blood derived substances in sequentially collected urine samples.

2.6.5. Uric acid excretion

In birds, although circulating plasma urate is freely filtered at the glomerulus, the majority (about 73%) is secreted in the proximal tubule. This involves active organic anion transport (OAT) from the blood at the basolateral membrane, followed by a cytoplasm to lumen step down an electrochemical gradient (Dudas et al., 2005; Dantzler, 2005). This OAT mechanism has relevance to the dramatic population crash of South Asian vultures from diclofenac poisoning (Green et al., 2006; Swan et al., 2006). Diclofenac is a non steroidal anti-inflammatory drug (NSAID), which in South

Asia was given routinely to debilitated farm animals. As vultures in this region commonly feed on fallen stock, which constitutes an efficient method of carcass disposal, these birds were consuming diclofenac from residues in the carcasses. In birds and other animals diclofenac inhibits the OAT mediated renal tubular transport of urate (Khamdang et al., 2002) and so this drug prevented uric acid being excreted by the vultures' kidneys. As a result blood uric acid concentrations became critically high in the birds leading to fatal visceral gout (Swan et al., 2006). Added to the nephrotoxic effect of diclofenac, this drug is also subject to enterohepatic recirculation in animals (Peris-Ribera et al., 1991).

Although urate secretion in the proximal tubule of the avian nephron is far from resolved, Dantzler, (2003) suggested it may involve vesicular cytoplasmic sequestration. Such a process may provide the initial nucleation step required for urate biomineralisation (Taylor and Simkiss, 1989). The hypothesis is that cytoplasmic vesicles, acting as condensing vacuoles (Mann, 2001), could provide a suitable confined reaction space for the formation of spherulitic urate. Such vesicles would then release their urate sphere contents into the lumen by exocytosis (Dantzler, 2003). Future research to answer the uncertainty over urate sphere formation would undoubtedly be useful in identifying their potential for avian urinalysis.

2.7. Urate spheres, a form of biomineralisation

2.7.1. Definition and example of biomineralisation

The process of biomineralisation is defined as the formation of biogenic crystals incorporating macromolecules that minimize structural anisotropic weaknesses, under physiological conditions (Weiner et al., 2000). This process, naming only a few, enables the biofabrication of bones, teeth, mollusc shells and fish otoliths (Mann, 2001). Otoliths, the least complex of these examples, grow in the inner ear of teleosts (bony fish), nourished by the endolymph, a gelatinous soup of solutes and protein. The constituents of the endolymph are both incorporated in and control the otolith structure (Tomas et al., 2004). As a result, changes in the environment or physiology of the fish, which affect endolymph constituents, are reflected in otolith chemistry (Halden et al., 2000; Thresher 1999). Otolith gromassh is characterised by alternating layers of protein and calcium carbonate, with the resulting temporal banding being used to age fish (Barker et al., 1997). The biomineralisation of otoliths depends on protein acid groups

creating localized sites of calcium ion super saturation (Strickland-Constable 1968). These sites induce nucleation (seeding) of vaterite spheres, which have a more charge dense crystal structure than calcite (vaterite: 6.7 Ca ions / nm^2, calcite: 4.5 Ca ions / nm^2). The same protein subsequently limits crystal gromassh of the spherulitic vaterite by surface encapsulation (Tong et al., 2004). A second protein creates the scaffold for these spherical building blocks to be laid down on, forming the gross structure of the otolith (Tomas et al., 2004). The otolith morphogenesis by virtue of these two proteins is consequently reported to be under genetic control (Sollner et al., 2003). Vaterite, a polymorph of calcite is an example of spherulite, which is defined as a form of abnormal crystal growth occurring under supersaturated conditions (Strickland-Constable 1968). Vaterite can form *in vitro* under the influence of organic acids (Tong et al., 2004; Grassmann and Lobmann 2004) or surfactants (Wei et al., 2004), which provide sites of high charge density; mimicking conditions created *in vivo* by the specific protein.

2.7.2. Avian urate spheres

From this description of otolith biomineralisation, several comparisons with avian urate spheres can be drawn. The glomerular filtrate in the proximal tubule may be compared to the endolymph, containing solutes and proteins. The proteins have a similar function in both instigating formation and limiting growth of the spherulitic urate. Furthermore, variations in the concentrations of solutes in the filtrate could be reflected in quantities incorporated within the urate spheres. It is reported that numerous elements present within the urate spheres are incorporated during their formation in the proximal tubule (Casotti and Braun, 2004). In vaterite formation, low protein concentrations promote sphere nucleation while high protein concentrations suppress crystal growth by forming a surface coat (Nys et al., 2004). Similarly avian urate spheres (Fig. 2.9.) have a central nidus of protein and an encapsulating outer surface of protein (Casotti and Braun, 2004).

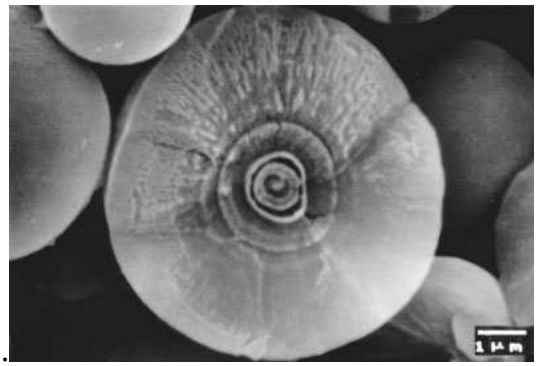


Figure 2.6. Section of urate sphere showing the laminated structure composed of a central protein nidus surrounded by 3–4 concentric narrow rings of protein the outer most forming the sphere surface (after Casotti and Braun 2004).

The alternating layers of protein and urate (Fig. 2.9.) may reflect their changing tubule concentrations, induced by glomerular filtration and tubular water reabsorption. However as urate spheres never form in the blood, even under the hyper uric conditions of visceral gout (Guo et al., 2005), the serum proteins present in the spheres (Janes and Braun, 1997) are unlikely to be responsible for their nucleation. Future research into resolving urate sphere formation in birds may be directed towards identifying the specific nucleation protein in the proximal tubule. Furthermore the gene responsible for such a protein may be common to all urate sphere-forming organisms.
Despite it being reported that biomineralisation requires specific physicochemical conditions for biogenic structures to form (Weiner et al., 2000), *in vivo* they commonly incorporate many contaminants. This is illustrated by chemical residues marking events in the formation of otoliths (Halden et al., 2000) and also avian bones taking up lead (Scheuhammer et al., 1999) or fluoride (Vikoren and Stuve 1995) from a bird's exposure to these pollutants. Likewise AUS are reported to have variable amounts of different ions randomly spread throughout them (Casotti and Braun, 2004) without apparently altering their structure.

2.7.3. Synthetic urate spheres

A further similarity with vaterite is that urate spheres can be synthesised *in vitro* (McNabb and McNabb, 1980). A simple method to achieve this is by cooling a saturated solution of uric acid in 1M sodium hydroxide (Fig. 2.7.). This process presumably depends on a high sodium ion concentration to replicate the high density

cation conditions necessary for spherulitic polymorph formation (Mann, 2001). Unlike the AUS which are restricted to <12μm in diameter (Braun, 2003), the *in vitro* spheres, produced in the absence of protein, typically reach 50μm in size (Fig.2.7.). While these artificial urate spheres are similar to AUS in being stable when re-suspended in absolute ethanol or after drying.

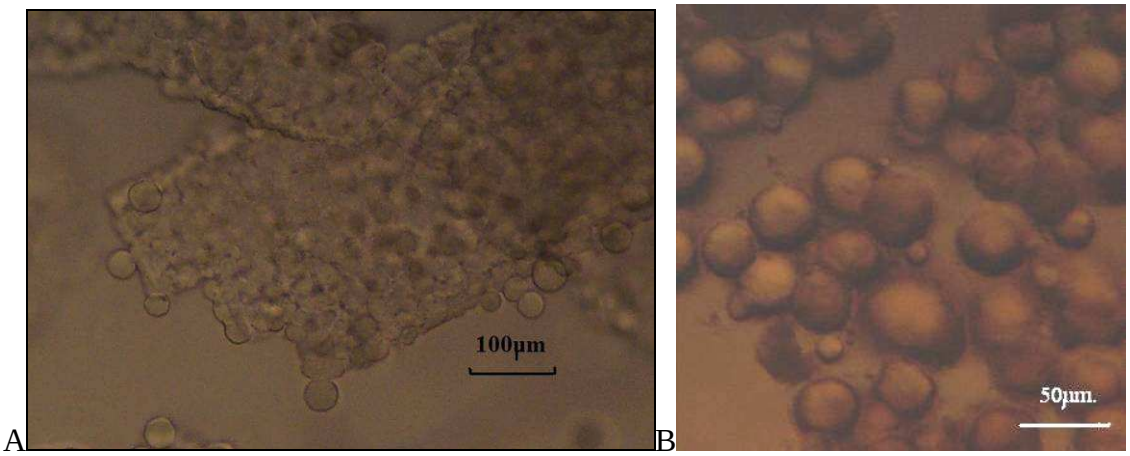


Figure 2.7. Spherulitic uric acid under medium power light microscope.
A: formation on the surface of flat plate-like crystals of normal uric acid
B: stable uric acid spheres re-suspended in ethanol.

2.8. Comparing the current avian urinalysis technique with using avian urate spheres to measure excreted metabolites.

2.8.1. Liquid avian urinalysis

Urine analysis (urinalysis) in avian species is becoming more widely used in a clinical context (Kurien et al., 2004; Tschopp et al., 2007), however only the liquid supernatant after centrifugal separation is analysed (Styles and Phalen, 1998). This centrifugation method disregards the potential value of analysing the solid urate spheres, which constitute the bulk of urinary excreted solutes (Casotti and Braun, 2004). Because this fluid portion may dry up or soak away, its application is limited (as in mammalian urine collection) to only captive or companion birds (Kurien et al., 2004). Such urinalysis is further restricted to samples of adequate volume, which may only occur in stressed birds or those with renal pathology (Harr, 2002). It is fortuitous that birds presented for veterinary examination typically pass wet polyuric droppings because of handling and transport stress (Styles and Phalen, 1998). However such diuresis would be expected to

have a marked diluting effect on urine parameters by reducing specific gravity and solute concentrations. As a result this could make quantitative interpretation of urine parameters problematic. Liquid urinalysis is reported in several bird species in the literature; including hummingbirds (Nicolson, 2005; Bakken and Sabat, 2006), starlings (Tsahar et al., 2005), pigeons (Halsema et al., 1988; Giladi et al., 1997), ostriches (Mushi et al., 2001), domestic chickens (Davis, 1927; Goldstein and Braun, 1989), companion birds (Styles and Phalen, 1998), and various falcons (Tschopp et al., 2007). Faecal contamination of urine samples is always a potential problem because of the shared cloacal outlet, which casts doubt on protein, glucose and blood concentrations in urine samples (Tschopp et al., 2007). To avoid such contamination renal catheterization, also called cloacal cannulation. has been employed in many experimental situations (Goldstein and Braun, 1989; Giladi et al., 1997). Such a procedure however is not favoured by clinicians, being highly stressful to the bird and requiring 10 to 30 min for collection (Styles and Phalen, 1998). For this reason the preferred method of urine collection is off a clean impervious surface of the bird's cage using a needle and syringe or a plain glass micro-haematocrit tube (Harr, 2002). The exception to this is urine collection from farmed ostriches where contamination can be avoided because, unlike other avian species, urine collects separately from faeces in the coprodaeum, which functions like a urinary bladder (Laverty and Skadhauge, 2008; Duke, 1999). On the analysis of the liquid fraction of ostrich urine, having no faecal contamination, Mushi et al., (2001) found that little if any detectable proteins or enzymes were present. A similar finding in falcons (Tschopp et al., 2007) further supports the questionable use of this technique for clinical diagnostics, while ignoring the great potential of urate sphere analysis. Because of the unusual nature of avian urine, Long and Skadhauge, (1983) warned against drawing conclusions about the renal excretion of substances in birds, if avian urine was analysed in a similar way to liquid mammalian urine. Clearly bird urine and mammalian urine are very different, requiring a completely different approach to their analyses.

2.8.2. Solid avian urinalysis

Although the urate spheres vary in diameter from <0.5µ to 12µ, their elemental composition in samples from individual birds are independent of sphere size (Casotti and Braun, 2004), allowing for gross samples of mixed sphere sizes to be analysed. However chemical constituents other than uric acid (65% dry mass.) and protein content

(5% dry mass.) can vary considerably between birds (Casotti and Braun, 2004). This contrasts with the finding that supernatant analysis of urine from healthy birds showed no significant biochemical differences between sex, age, species or the fasted and postprandial states (Tschopp et al., 2007).

Casotti and Braun, (2004) concluded that the chemical composition of urate spheres bore no relationship to the dietary preferences of different bird species. However their data showed a significantly higher calcium concentration in the urate spheres from domestic chickens compared to various wild birds. This difference may relate to commercial poultry diets being fortified with this element, typically having dry mass calcium concentrations of over 3.5% (Safaa et al., 2008). Although the avian kidney reabsorbs more than 98% of filtered calcium (Wideman, 1987), experimentally elevating plasma calcium concentrations increases renal calcium excretion in birds (Clark et al., 1976). In domestic fowl there is a dramatic change in urine composition when the egg is laid. Use of the technique of energy dispersive x-ray analysis (EDAX), similar to that reported in this thesis, has demonstrated that in addition to turning alkaline, the urine also contains appreciably greater quantities of calcium (Sykes, 1971). Higher concentrations of calcium were detected in urate spheres from five out of six laying chickens fed a 4% calcium diet (Janes and Braun, 1997) compared to concentrations detected in five (mixed sex) poults (Casotti and Braun, 2004). As egg laying birds typically have elevated plasma calcium compared to non-laying birds (Dacke, 2000) these findings suggest calcium concentrations in urate spheres may reflect blood concentrations.

2.8.3. Proteins in avian urine

Compared to normal human urine which typically has <0.05 mg/mL of protein, avian urine (the combined liquid and solid) contains 5mg/mL of protein (Braun, 2009). This plasma-derived protein (Janes and Braun, 1997) is almost entirely associated with the urate spheres (Harr, 2002), making them 5% protein by dry mass (Braun and Pacelli, 1991).

Although Janes and Braun, (1997) reported that the relative protein concentrations in the urate spheres differed slightly from plasma concentrations, this may have resulted from several factors. These include (a) variation in tubular re-absorption of different sized proteins, (b) the addition of glycoproteins (McNabb et al., 1973; Mirabella et al., 1998) similar to mammalian Tamm-Horsfall proteins (Serafini-Cessi et al., 2003) or (c)

the effect of sampling stress in the birds (Styles and Phalen, 1998). The latter possibility arises because the plasma samples were taken at least 10-30 min after urine was collected by catheterization, a very stressful process for birds (Halsema et al., 1988). Stress induces diuresis (Styles and Phalen, 1998), so diluting the urine proteins and altering plasma protein concentrations (Grasman et al., 2000). Because of the inevitable time lag between urine formation and its passage (the time urine takes to pass from the glomerulus to the urodeum), it may have been more appropriate to collect plasma samples before catheterization for comparison. However despite such potential causes for variation there is a striking similarity between plasma and urate sphere proteins as reported by Janes and Braun, (1997).

The molecular mass of proteins present in the spheres is reported to be restricted by the dimensions of the glomerular pores (Casotti and Braun, 1996), with the six most abundant proteins being between 30 and 149kDa (Janes and Braun, 1997). This would preclude the inclusion in urate spheres of larger plasma proteins, like gamma globulins (160kDa) and vitellogenin (200kDa), while allowing smaller binding proteins from plasma such as corticosteroid binding globulin (50-60kDa), prealbumin (6.1kDa) and metallothioneins (4-14kDa) to be included.

As albumin, the most abundant protein in AUS, is derived from the bloodstream (Janes and Braun 1997), AUS analysis should reflect concentrations of the wide range of substances bound to this protein in the blood, which includes hormones, metabolites and toxins (Peters, 1996). In addition, physicochemical alterations of the albumin molecule itself have been shown to be a valuable measure of systemic oxidative damage in man. These include ischaemic modified albumin (Roy et al., 2004) and reduced albumin cobalt binding (Bar-Or et al., 2001) in blood samples. Analysis of another albumin type, egg white, has also shown great potential for 'protein finger printing' in ecological studies (Andersson and Ahlund, 2001). Other blood derived proteins found in the urine of man and other animals are markers of systemic disease (Zimmerli et al., 2008). For example the presence of metallothioneins in urine following cadmium exposure (Shaikh and Tohyama, 1984) and aminolevulinic acid associated with lead poisoning (Sithisarankul et al.,1999; Fukui, 2005; Buttery, 1995). The small zona radiata protein (50kDa), a biomarker of endocrine disruption in birds (Jimenez et al., 2007), may also pass into the AUS from the blood stream.

It must be noted that uric acid also has significant binding properties (Mikulski et al., 1994; McNabb & McNabb, 1980), further adding to the host of substances which potentially may be incorporated into the urate spheres during formation.

2.8.4. Non-protein constituents of avian urine

In common with mammalian urine many chemicals, including body metabolites, are excreted in avian urine (Casotti and Braun, 2004; Rettenbacher et al., 2004; Lepschy et al., 2008). However, unlike in mammals, most solutes are not in solution but reside bound in the urate spheres, having been incorporated during sphere formation in the proximal tubule (Casotti and Braun, 2004). The elemental composition of the AUS has been reported for several bird species by Casotti and Braun, (2004). Nitrogen is the most prevalent element found in AUS, derived from the uric acid and protein content. Potassium is typically the next most prevalent element in the spheres over a wide range of bird species. Other elements present in smaller amounts, are calcium, sodium, potassium, sulphur and chlorine. Other than nitrogen these elements vary in proportion between species, while such concentrations are relatively independent of sphere size in any one sample. Furthermore, unlike the protein which is arranged in discrete concentric layers, the individual elements are randomly distributed throughout the sphere (Casotti and Braun, 2004). These reported variations in the non-protein content of AUS may suggest that such differences between birds could reflect dietary preferences.
To date specific compounds such as hormones have not been identified within the AUS.

2.8.5. Urate sphere physicochemical properties

AUS are stable in dry conditions and may even be preserved in arid or covered archaeological sites (Canti, 1998). Normally, however, they disaggregate on wetting then re-crystallize as euhedral, bladed or lenticular crystals of uric acid dihydrate (Fig.2.8), with the consequent release of trapped electrolytes, especially potassium (Drees and Manu, 1996).

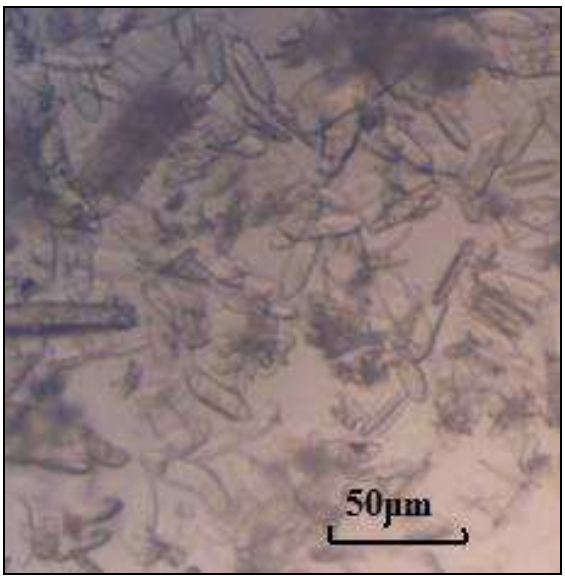


Figure 2.8. Re-crystallised domestic chicken urate spheres (after 15mins at room temperature in an acetate buffer pH 4.6), having a similar appearance to those described by Drees and Manu, (1996).

Acid conditions can accelerate this process (Drees and Manu, 1996) while alkaline solutions, especially lithium carbonate, may fully dissolve the urate spheres without re-crystallization (Adeola and Rogler, 1994). In addition to desiccation, freezing preserves the intact urate spheres (personal observation). Also suspension in ethanol or acetone (Drees and Manu, 1996) causes no visible morphological disruption. These methods of preservation would also prevent faecal uricolytic organisms degrading the urate spheres (Braun, 2009). Such organisms are abundant in the avian lower bowel where they play an important part in the nitrogen recycling of refluxed urine (Braun, 2003).

2.9. Conclusions

Birds have a valuable status as sentinels of environmental change (Peakall, 2000), which combined with their ability to produce collectable solid urine samples should make them ideal biomonitors (Fossi, 1994). However this chapter has highlighted some problems, which may need to be resolved before avian urate spheres (AUS) can be used for urinalysis in birds. These include the enterorenal recirculation of excreted metabolites, resulting from the combined effects of urine reflux and the renal portal system (Laverty and Skadhauge, 2008). Although this process may only relate to substances resistant to degradation in the lower bowel, their identification could be difficult and stability may not be consistent. Furthermore a high proportion of excreted

urine may be uncollectable because the refluxed urate spheres are broken down in the lower bowel (Braun, 2009). The amount of urine degraded in this manner varies between species and even within individuals depending on their state of hydration (Laverty and Skadhauge, 2008). As a result substances intermittently excreted in the urine may not be consistently detected in urate sphere samples.

Despite these reservations the following chapters report on several studies to identify biologically relevant compounds excreted in the urate spheres of various bird species with the aim of showing the analysis of AUS is suitable as a non-invasive biomonitoring method for environmental pollution and stress.

Chapter 3.

Using domestic chickens (Gallus gallus domesticus) to biomonitor a heavy metal contaminated soil

3.1. Introduction

Metals are natural substances, but their increased availability through mining and smelting, with subsequent release into the environment from numerous anthropogenic sources, makes them a serious threat as persistent toxic pollutants (Walker et al., 2001). Some metals (copper (Cu) and zinc (Zn)) are essential trace elements but are toxic at higher concentrations (Sileo et al., 2004), while others (e.g. lead (Pb) and cadmium (Cd)) have no biological function and can be toxic at low concentrations. High concentrations of Cu and Zn are commonly found with other metals in mine waste and their toxic impact on fauna, including wild birds, is illustrated by the 1998 mine waste spill in Spain's Donana National Park (Gomez et al., 2004). Pb poisoning of free-ranging birds following incidental uptake from spent fishing weights and gun shot is also well recognised (Scheuhammer and Norris, 1996). The widespread use of metals in the world makes it necessary to monitor their concentrations in the environment to safeguard biological systems. Typically metals accumulate in soil and sediment where they can exist in many states, which determine their bioavailability (Ruby, 2004). For this reason a measure of their biological uptake through biomonitoring techniques is more relevant than total environmental concentrations (Peakall and Burger, 2003). Birds are widely used for biomonitoring environmental heavy metals (Furness, 1993), with most tests involving invasive or destructive sampling techniques (Swaileh and Sansur, 2006). The advantages of using non-destructive strategies in biomonitoring programmes (Fossi, 1994) have led to the increasing use of eggs (Burger and Gochfeld, 1993), feathers (Burger et al.,, 1992) and guano (Fitzner et al., 1995), to biomonitor avian exposure to environmental heavy metals. In order for such materials to be valid for metal biomonitoring they need to correlate with blood concentrations (Hollamby et al., 2006), representing the bird's current bioavailable intake (Furness, 1993). Compared to the acquisition of eggs from wild birds being restricted to the laying season and feathers to the time of moult, whole guano has the advantage of continuous production and easy collection.

Guano is a mixture of faecal and urinary excretions resulting from the digestive and urinary systems sharing a common outlet in birds called the cloaca. The faecal

component of guano contains a variable mixture of bile excreted (representing bioavailable) and unabsorbed heavy metals simply transiting the digestive system (Mohanna and Nys, 1998). The white, urine component is derived from the blood passing through the kidneys (Casotti and Braun, 2004). Consequently its heavy metal content should be linked to the bird's current 'bioavailable' intake (Furness, 1993) i.e. the metal has been taken up into the blood stream from the environment.

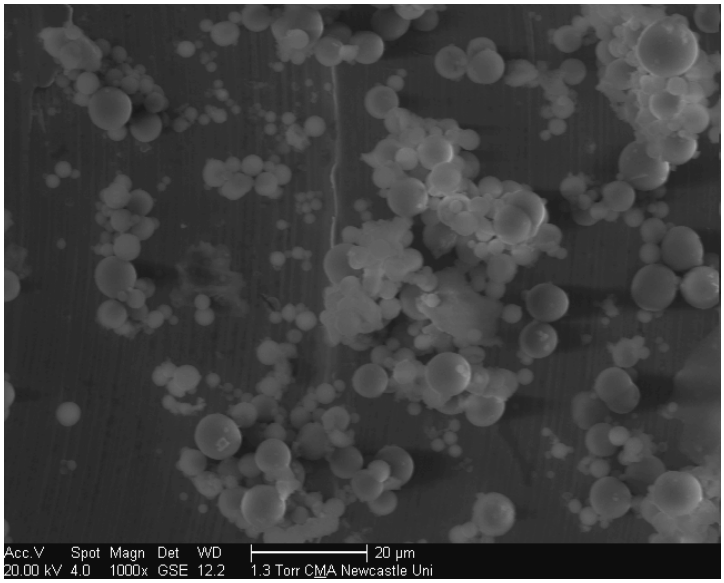


Figure 3.1. Scanning electron micrograph (SEM) of ethanol extracted avian urate spheres (AUS) from chickens on the contaminated allotment.

Avian urine is a suspension of microscopic biomineralised (Taylor and Simkiss, 1989) uric acid particles (Fig.3.1) called urate spheres (Casotti and Braun, 2004). These avian urate spheres (AUS) have significant ion binding properties (McNabb & McNabb, 1980), being composed of 5% protein by dry mass (Braun and Pacelli, 1991). The predominant protein in AUS is serum albumin (Casotti and Braun, 2004), which is recognised to bind heavy metals (Bal et al., 1998). The AUS is also 65% by dry mass uric acid, which binds several transition metals (Mikulski et al., 1994). Thus it is likely that, during formation in the avian kidney, the AUS will incorporate heavy metals filtered from the blood stream. In this way AUS make the urine component of guano useful for measuring heavy metal exposure in birds because they may bind and package metals for urinary excretion. It can therefore be hypothesized that excess uptake (to the chickens' requirements) of essential metals Cu and Zn will be reflected in their increased urinary excretion, as part of homeostatic regulation, while non-essential

metals such as Pb, will be excreted in direct proportion to blood concentrations. Further support for using AUS to monitor metal concentrations is that human urine is used for biomonitoring exposure to Pb, of which approximately 70% is excreted via the urine (C.D.C., 2005). Urinary excretion of heavy metals in mammals is dependant on metallothioneins, which avidly bind Zn, Cu, Cd, Pb and Hg, enabling their transport, detoxification and metabolism in the body (Nordberg, 1998). As metallothioneins also play a major role in metal excretion in birds (Nam et al., 2005), and their molecular size (6.5 kDa) would allow passage in glomerular filtrate (Janes and Braun, 1997), avian urine is highly likely to contain heavy metals.

The overall aim of the work in this Chapter is to demonstrate that AUS contain measurable concentrations of heavy metals, which reflect a bird's exposure to contaminated soil and thus can potentially be used as a sampling technique for biomonitoring environmental heavy metal contamination.

3.2. Materials and methods

3.2.1. Background

To determine the potential of this avian urine-based technique for measuring metal bioavailability from a contaminated soil, in 2006 -2007, I was able to sample birds on a local Newcastle City allotment (Branxton) prior to and following its remediation. Laying chickens were kept on the allotment, which had a soil known to be contaminated with Pb, Cu and Zn (Pless-Mulloli et al., 2004). These chickens provided an ideal sentinel species (Peakall and Berger, 2003) for biomonitoring the soil heavy metal contamination, before and six months after site remediation by top soil replacement. As the chickens had access to the contaminated soil, and ingested soil may typically constitute 10% of their diet (Beyer et al., 1994), these birds were expected to have elevated body metal concentrations before soil remediation.

As a control, metal concentrations in AUS from laying chickens on a similar Newcastle City allotment (Walker Road), where no soil exposure was allowed, were compared with concentrations in urine from birds on the contaminated allotment. The proposed urine based method was also compared with commonly used non-destructive sampling

techniques (egg, feather and whole guano analysis) for heavy metal biomonitoring using birds.

Figure 3.2. Photographs of the chickens on the contaminated allotment A: before and B: after remediation. The only difference in the chicken pen being that the soil beneath the feeder had been replaced. In contrast, the difference outside the chicken pen between C: before and D: after remediation was more obvious.

3.2.2. Sample collection

Samples were collected from the contaminated allotment on 2nd November 2006, a few days prior to remediation, and subsequently six months later on 25th April 2007. The samples from the control allotment were collected on 10th November 2006.

Site metal concentrations and soil sampling

Soil samples were collected for metal analysis in order to confirm the contamination status of the allotment. It was previously reported as having geometric mean soil concentrations for Cu, Pb and Zn of 166, 674 and 823 mg kg^{-1} (dry mass) respectively (Hartley et al., 2004).

Six surface horizon (0-10cm) soil samples were taken from a chicken pen on the contaminated allotment to determine total soil heavy metal concentrations available to the chickens. The soil samples were kept refrigerated (0-4^{0}C) in sealed plastic bags until processed (see 3.2.4). It was not necessary to take soil samples from the control allotment because the birds here were kept entirely on wood shavings with no access to soil. Six months after site remediation samples of the new soil were taken from the rebuilt chicken pen in which the birds previously exposed to contaminated soil were kept.

Biomonitor sampling

Background: Chicken housing and feeding regimen

On the contaminated allotment, prior to remediation, the chickens were kept in a group of 20 birds confined to a caged enclosure (approximately 6m^2) with a floor of exposed contaminated soil, where they were fed (Fig.3.2). The birds had an adjoining separate night roost consisting of a wooden floored shed with perches and nest boxes. The diet of these birds was a commercial brand fed *ad libitum* consisting of a mixture of pelleted feed and whole grains. Also varying amounts of kitchen scraps (vegetable peelings and stale bread) were fed. Fresh water was provided in drinkers replenished daily with tap water from the domestic supply.

On the control allotment a single group of 35 chickens was housed in a purpose-built shed having a concrete floor covered with regularly replenished wood shavings. The birds were exclusively fed *ad libitum* on a pelleted commercial laying ration (different from that fed on the contaminated allotment) and water was provided from a domestic supply. Birds were not allowed access to any soil on this control allotment.

Six representative samples of the rations as fed (excluding kitchen scraps) were collected from each allotment for metal analysis; the exact same ration was fed before and after remediation to the chickens on the contaminated allotment.

Samples collected for biomonitoring

From the night roost and nesting boxes on both the contaminated and control allotments several kilograms of freshly passed guano, a few freshly laid eggs (contaminated: n=3, control: n=6) taken to have been laid by different hens and numerous feathers were collected. Following remediation of the contaminated site only guano was collected for analysis. Eggs were not collected because of the low metal levels recorded. Feathers

were not collected either because they were expected to still reflect earlier exposure being shed only annually (King and McLelland, 1975).

Sample preparation

Eggs

The eggs were thoroughly cleaned using warm tap water and a nylon brush, rinsed with 18MΩ deionised water and then dried prior to being stored frozen (-20^{0}C). To prepare the eggs for metal analysis the shell was peeled from the frozen eggs and internally adherent albumin washed off with18MΩ deionised water. The albumin was discarded, this being facilitated by it thawing quicker than the yolk, which was retained for analysis. The albumin was not analysed because of its reported low affinity for Pb and Cu in chickens exposed to elevated intake concentrations (Flores et al., 1997; Skrivan et al., 2006).

Feathers

Clean, intact wing primary feathers were collected and grouped by their colour into four samples corresponding to the chicken breeds on the contaminated allotment (Rhode Island Red, Moran, White Leghorn and Black Rock). The feathers from the control allotment had only three different colours so provided just three sample groups for analysis. Although not guaranteeing feathers came from different individual birds different colours could be assumed to not be from the same one and so give some measure of biological variation. Feathers were first washed with warm tap water, then thoroughly rinsed with 18MΩ deionised water and dried before storage at room temperature in sealed plastic bags. This simple cleaning method was chosen because despite repeated acetone and water washes, favoured by many authors (Burger et al., 1992), feathers still appear to retain the heavy metals accumulated from external contamination (Dauwe et al., 2003).

Guano

Only fresh, whole guano pellets were selected with any adherent feathers or bedding material removed prior to storing frozen (-20^{0}C) in sealed plastic bags.

3.2.3. Urate sphere extraction and uric acid analysis of AUS

Background

Avian urine is composed of a colloidal suspension of discrete spherical urates measuring 0.5 to 10µm (Casotti and Braun, 2004), these spheres are insoluble in ethanol or acetone but are disrupted in aqueous solutions (Drees and Manu, 1996). The extraction technique depended on the principle of differential sedimentation; the small size of the urate spheres allowing them to remain suspended in the ethanol for longer than larger or denser particulates including soil from the faecal component.

Extraction

To extract the urate spheres from whole guano, an approximately 200g representative sample of frozen guano was defrosted at room temperature with 300mL GPR absolute ethanol in a glass beaker. On breaking the guano up with a glass rod, the white urinary component readily formed a suspension, any gross floating faecal contaminants being removed at this time. To avoid the transfer of denser faecal material including soil particulates, only the upper portion of the supernatant was decanted into a 50mL glass test tube. The supernatant was allowed to settle for 5 min, after which the top 20-30mL portion (representing the extracted urate sphere sample) was decanted into a 50mL polypropylene centrifuge tube (Fisherbrand®). The residue from the glass test tube was returned to the beaker and the process repeated 4-5 times until no further white urate spheres could be extracted. The extracted urate sphere sample was centrifuged at 2000 x g for 2 min, the discoloured ethanol discarded and the solid AUS washed twice with approximately 40mL of fresh ethanol by vortexing and again centrifuging (2000 x g for 2 min).

Quantification of AUS contents

Qualitative purity of the solid urate sphere extract was determined by examining a small representative fraction under a medium power light microscope, an adequately pure sample consisting almost entirely of characteristic urate spheres (Fig. 2.1A).

To quantify the metal constituents in the AUS extracts it was necessary to determine their uric acid content. This was achieved by using a combination of two methods for uric acid analysis in avian guano (Van Handel, 1975; Adeola and Rogler, 1994). In brief, an accurately weighed 30-40mg representative fraction of the dry extracted urate

spheres was digested in 10mL of 0.5% (w/v) $LiCO_3$ solution in a boiling water bath for 10min. The resulting solution was made up to 100mL with 18MΩ deionised water, from which a well-mixed representative 5mL sample was filtered using a 0.45μm pore size syringe filter (Whatman® Puradisc™ 25 AS). A 100μL aliquot of this filtrate was diluted to 1mL, added directly to a cuvette and mixed with an equal volume (1mL) of the freshly made up chromagen reagent (Van Handel, 1975). Although the original Van Handel (1975) method used a 5 minute end point, it was found 10 min was preferable, at which time the absorbance (450nm) of the yellow product was measured using a Biochrom Libra S12 UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, UK). Calibration was achieved using water blanks and uric acid standard solutions made up in 0.5% (w/v) $LiCO_3$. Specificity to uric acid was determined by overnight incubation at room temperature of sample duplicates with an equal volume of 0.5U/mL uricase (Sigma), which resulted in equivalent to blank readings.

Using protein to quantify AUS contents

After uric acid, protein is the next most prevalent constituent of the AUS, predominantly serum albumin (Casotti and Braun, 2004). As a consequence of this it was proposed that protein content may be used to quantify the urate sphere constituents. Because the acid conditions used to break open the AUS would hydrolyse proteins into their amino acid components, an alternative method to extract the protein content of AUS was explored. The method by Sharif and O'Hagan, (1995) using 5% w/v SDS in 0.1 M sodium hydroxide successfully dissolved the AUS, liberating the protein into solution. Replicating work by Janes and Braun, (1997), the Bradford dye method (Bradford, 1976), using premixed reagents (Bio-Rad Protein Assay), was used to measure the AUS protein content. However it was found impractical to measure the released protein because it co-precipitated with the uric acid. This was because uric acid has a high affinity for proteins such as serum albumin (McNabb and McNabb, 1980). To date the chemical constituents of extracted urate spheres have only been quantified against uric acid content, although if a suitable method for protein analysis can be devised the protein content may be used as an alternative.

3.2.4. Sample preparation and analysis

All soil, chicken feed, egg, feather, whole guano and extracted urate spheres were oven dried to constant mass at 65^0C, then ground and sieved to homogenise, prior to taking a representative sample for heavy metal analysis. Pb, Cu, and Zn concentrations for all samples other than chicken feed and those collected following remediation, were determined by a UKAS (United Kingdom Accreditation Service) accredited laboratory using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after a 2 hour acid reflux pre-digestion. Concentrations of detection were poorer for some of the smaller samples but generally were between 2 and 8mg kg^{-1} for Cu and Pb while >0.8mg kg^{-1} for Zn on a dry mass basis. Metal analysis of chicken feed (from both allotments), whole guano, extracted urate spheres and soil (from the remediated allotment) was performed using a Unicam 701 ICP-OES (Unicam Instruments, Cambridge, England) after acid reflux using a standard method (EPA, 1991). Calibration standards (Sigma) were freshly made up for each metal, having limits of detection in a complicated matrix solution of 0.1mg L^{-1} for Pb and 0.01 mg L^{-1} for Cu and Zn. The metal concentrations in the two acids were determined by digesting acid blanks in the same manner as the samples, this value being subtracted from the sample results before calculating metal values. The resulting limits of detection in dry samples for Pb, Cu and Zn were 5, 0.5 and 0.5 mg kg^{-1} respectively.

All metal concentrations are expressed as mg kg^{-1}on a dry mass basis, with urate sphere concentrations as μg g^{-1} uric acid, allowing for sample comparison to compensate for variations in extraction purity.

Representative samples of the dried, ground and sieved (2mm) soil were analysed for pH and soil organic matter (SOM) using a modified method (Clark et al., 2006) because these properties have a strong influence on the bioavailability of soil heavy metals (Ruby, 2004). The pH_w was determined after reacting soil for 1 hour, 1:1 with 18MΩ deionised water (5g/5ml). The pH_w of the supernatant was measured, after centrifuging (2000g for 2mins), using a pre-calibrated glass electrode (Denver Instruments). The SOM content was determined on accurately weighed 5g soil samples by mass reduction from overnight ignition in a 450^0C furnace.

3.2.5. EDAX analysis of extracted urate spheres

The opportunity arose to use the facilities of the environmental electron microscope in the Advanced Chemicals & Materials Analysis Service at Newcastle University. This unit can analyse small particulates similar to the urate spheres for their elemental constituents using energy dispersive x-ray micro-analysis (EDAX) (Vesk and Byrne, 1999). Furthermore this method of analysis has previously been used to identify the elemental composition of urate spheres from birds, including domestic chickens (Casotti and Braun, 1997; Casotti and Braun, 2004).

3.2.6. Data analysis

Statistical analyses were not performed on the whole guano and AUS data because bulked samples had been used, giving effectively n=1, consequently sampling would reflect technical rather than biological variability. Data from the small number of individual egg and feather samples was analysed using the non-parametric Mann-Whitney U-test. For these, sample results are reported as median and range values. However the larger number of soil and feed samples (being normally distributed) allowed parametric analysis using the Student's t-test, with significant differences ($p< 0.05$) set at a 95% confidence interval. Where samples were below the limit of detection (LOD), a value of half the LOD was used in data analysis (Nicholson et al., 1999; Dauwe et al., 2005).

3.3. Results

3.3.1. Soil analysis

Soil samples from the chicken pen on the contaminated allotment prior to remediation had elevated concentrations of Pb, Cu and Zn (Table 3.1) when compared with background values reported in the literature (McGrath and Loveland, 1992). These elevated concentrations were comparable to previous results reported for this allotment (Hartley et al., 2004). The mean metal concentrations of the soil samples from the same pen after remediation were similar to reported background concentrations (McGrath and Loveland, 1992). However this fresh soil had significantly ($p<0.05$) lower concentrations of all three metals in comparison to the pre remediation pen soil.

Both the contaminated and replacement soils had neutral pH values (7.3±0.3 and 7.31±0.03 respectively) confirming earlier reported values (Hartley et al., 2004). The higher SOM value of 33.5±10.2% in the contaminated soil compared to 7-13% for allotment soils reported in the literature (Clark et al., 2006), may be related to its dark colour and visible residues of chicken manure. The replacement soil was lighter coloured, with a more clay-like appearance and had a lower SOM value (12.3±0.5%) despite some visible feed and guano content.

Table 3.1. Top soil pH, percentage soil organic matter (%SOM), lead (Pb), copper (Cu) and zinc (Zn) from the chicken pen on the contaminated allotment before and after remediation by soil replacement, compared with previous data for the allotment soil and mean background values for soils in England and Wales reported in the literature.

		pH_w	**%SOM**	**Pb**	**Cu**	**Zn**
Chicken pen soil	Preremediation	7.30±0.30	33.5±10.2	555±301	273±59	827±241
	Postremediation	7.31±0.03	12.3±0.5	58.5±10.8	15.1±1.1	57.7±2.3
Soil concentrations prev. study[a] (n=12)		7.3±0.2	N/R	674±286	166±76	823±194
Mean background soil concentrations				74	23	97

Metal concentrations as geometric mean ± sd mg kg^{-1} dm (n=6).
[a]Hartley et al., (2004).
[b]McGrath and Loveland (1992).

3.3.2. Chicken feed analysis

Lead concentrations in chicken feed samples from both allotments were not significantly different from each other ($p>0.05$, 95% CI) and comparable to the reported low concentrations in commercial poultry feeds (Table 3.2). Both Cu and Zn concentrations were significantly different ($p< 0.01$, 95% CI) between feed given on each allotments. The control chicken feed was at the low end of reported Cu and Zn concentrations in the literature, however in contrast the contaminated allotment feed concentrations were well below this (Table 3.2).

On gross examination of the two feed samples it was clear the chickens on the contaminated allotment were fed on a ration containing a high proportion of whole grain, compared to the control chickens' diet, which consisted entirely of pelleted feed.

Table 3.2. Lead (Pb), copper (Cu) and zinc (Zn) concentrations (mg kg^{-1}dm median and range) in chicken feed as fed on the contaminated (pre and post remediation) and control allotments, with for comparison the reported range of values in home mixed feed fed to laying chickens in England and Wales.

Feed samples	Pb	*Cu	*Zn
Control allotment	1.03 (0.75-1.84)	10.04 (9.65-13.28)	57.05 (80.33-53.66)
Contaminated allotment	1.50 (1.19-1.66)	5.83 (5.11-6.12)	18.72 (17.32-23.71)
Reported range of values[a]	<1-1.12	10.7-56.1	94.1-311

Metal concentrations as median and range mg kg^{-1} dry matter (n=6).
Asterisks indicate significant difference p<0.01 at 95% CI (Mann-Whitney U-test) between feed from control and contaminated allotments.[a]Nicholson et al., (1999).

3.3.3. Metal concentrations in eggs (yolk and shell)

Pb values in all egg samples (yolk and shell) from both allotments were below the level of detection (LOD) of 2mg kg^{-1} dry mass (Table 3.3). Zn concentrations in the egg yolks were not significantly different (p>0.5 at 95% CI) between the contaminated allotment and control site. However, concentrations of Cu in the yolks from the contaminated site were significantly (p<0.05 at 95% CI) higher than control samples. The Zn concentrations in shell samples from chickens on the contaminated site were significantly (p<0.05 at 95% CI) higher than control site egg shell values. Cu concentrations in egg shell samples were below the LOD of 2mg kg^{-1} dry mass in all control eggs and two of the three contaminated samples making comparison impossible.

Table 3.3. Lead (Pb), copper (Cu) and zinc (Zn) concentrations in chicken egg samples (yolk and shell) from chickens on the contaminated (prior to remediation) and control allotments.

Egg samples		Yolk			Shell		
		Pb	Cu	Zn	Pb	Cu	Zn
Contaminated allotment (n=3)	1	<2	3	68	<2	<2	<4
	2	<2	3	70	<2	<2	7
	3	<2	3	94	<2	2	<4
Control allotment (n=6)	1	<2	2.9	73	<2	<2	1.8
	2	<2	<2	52	<2	<2	0.9
	3	<2	<2	77	<2	<2	1.7
	4	<2	2	74	<2	<2	<0.8
	5	<2	2.2	78	<2	<2	1.2
	6	<2	<2	73	<2	<2	<0.8

<2, <4 or <0.8 signifies value was below the relevant limit of detection of 2, 4 or 0.8mg kg^{-1}

Values as mg kg^{-1} dry mass.

3.3.4. Metal concentrations in feathers

Chicken feathers from the contaminated allotment (Table 3.4) had significantly ($p<0.05$) greater Pb concentrations than the control birds assuming the control birds to have concentrations at half the LOD. However Zn concentrations in feathers from the contaminated allotment were not significantly increased over controls. Conversely there was significantly ($p<0.05$) more Cu in feathers from control birds compared to feathers collected from birds on the contaminated site.

Table 3.4. Lead (Pb), copper (Cu) and zinc (Zn) concentrations mg kg^{-1} as median (and range) in feathers from chickens on the contaminated and control allotment.

Feather samples	Pb	Cu	Zn
Control site (n=3)	<8	17 (16-23)	130 (100-130)
Contaminated site (n=4)	14.8 (13-18)	9.5 (8-10)	140 (120-240)

3.3.5. Metal concentrations in whole guano

Whole guano from chickens on the contaminated allotment (Fig.3.5) before remediation had higher median Pb concentrations than the guano collected from the control site; median Cu and Zn concentrations however were not obviously different. All three metal concentrations in the guano from chickens on the contaminated allotment prior to remediation were higher than in the guano of the same birds 6 months after remediation. In comparison with control site metal concentrations, the guano from the remediated site appeared to have higher Pb but lower Cu and Zn.

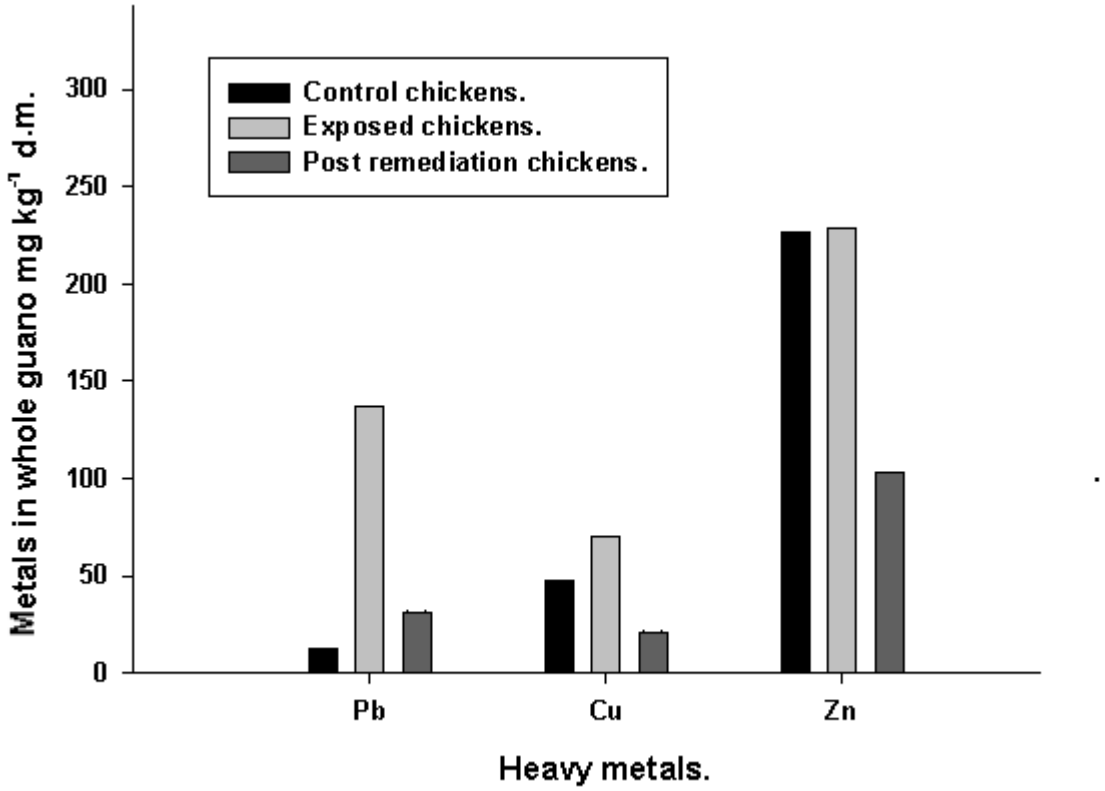


Figure 3.3. Median (n=6) metal concentrations (mg kg^{-1} dry mass) in whole guano. Lead (Pb), copper (Cu) and zinc (Zn) concentrations in samples from exposed chickens before and after soil remediation compared to unexposed control chickens.

3.3.6. AUS analysis

Fewer samples were analysed because several were lost as a result of the high reactivity of the extracted AUS with acid during the pre-digestion stage of analysis. This resulted in there only being two analyses from exposed chickens and four each from control and post remediation chickens.

3.3.6.1. Purity of AUS samples

Representative samples of extracted AUS were examined by light microscopy and all appeared to be free of contamination from soil or faecal material.
The chemical analysis of each sample of extracted AUS ranged from 54 to 60% uric acid by dry mass, representing a purity of 83 to 92% respectively.

3.3.6.2. AUS metal concentrations

The extracted AUS samples from the chickens on the contaminated allotment prior to remediation appeared to have elevated concentrations of all three metals compared to AUS samples from chickens on the control allotment (Table 3.5). Six months after remediation the median concentrations of Cu and Zn were 16 and 85 μg g^{-1} uric acid respectively being below both control site and pre-remediation values. The median Pb concentration of 147 μg g^{-1} uric acid post remediation was similar to the pre-remediation concentrations while still higher than control concentrations.

Table 3.5. Lead (Pb), copper (Cu) and zinc (Zn) concentrations μg g^{-1} uric acid as median (and range) in extracted urate spheres from chickens on the contaminated (pre- and post-remediation) and control allotments.

Sample source		**Pb**	**Cu**	**Zn**
Contaminated allotment	Pre-remediation (n=2)	208.5 (208-209)	65.8 (65.6-66.1)	526 (512-540)
	Post-remediation (n=4)	147.0 (118-413)	16.0 (15-19)	84.5 (84-102)
Control site (n=4)		6.6 (4.7-7.5)	37.6 (17.7-49.1)	250.3 (135.5-315.8)

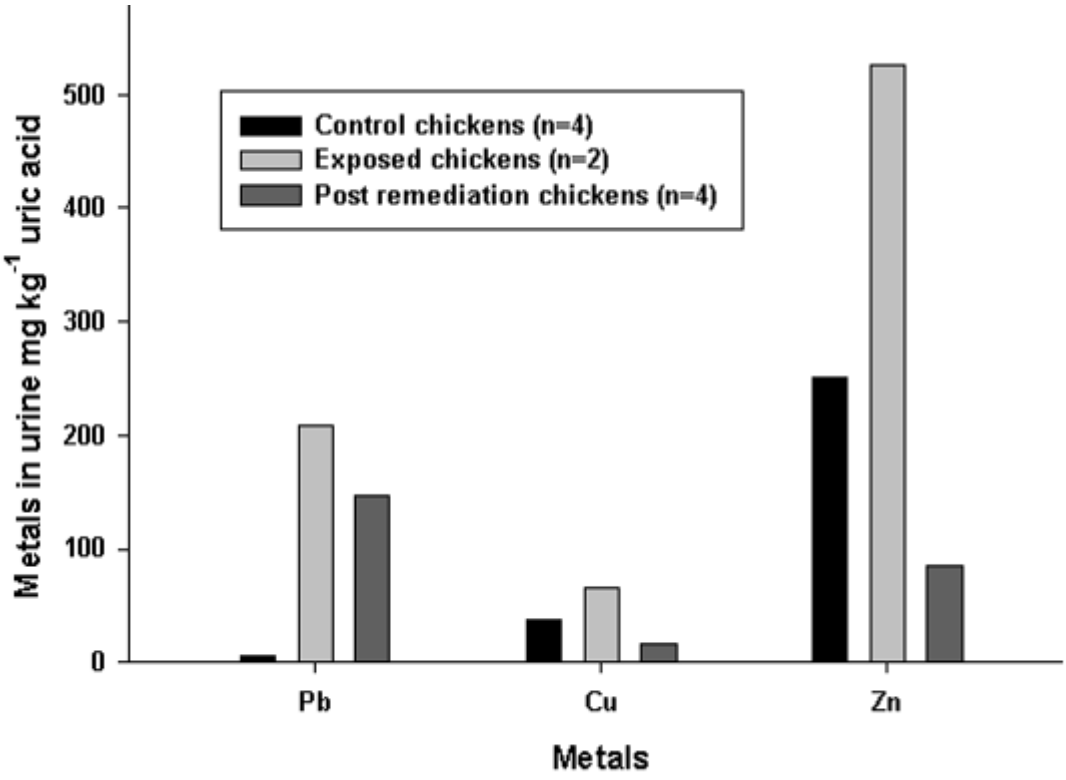


Figure3.4. Bar chart of median Lead (Pb), copper (Cu) and zinc (Zn) concentrations (μg g^{-1} uric acid) in extracted urate sphere (urine) samples from exposed chickens before and after soil remediation compared to samples from unexposed control chickens.

3.3.6. 3. EDAX analysis of extracted urate spheres

The percentage dry mass content of Pb, Cu and Zn determined by ICP-OES even in the contaminated birds were all below the EDAX detection limit of 0.1% dry mass. As a result these metals were not expected to be detected in the EDAX analysis (Fig.3.5B). However Ca and K were detected by this method, a consequence of both ions being typically between 2 and 5% by dry mass of urate spheres using ICP-OES analysis (personal findings).

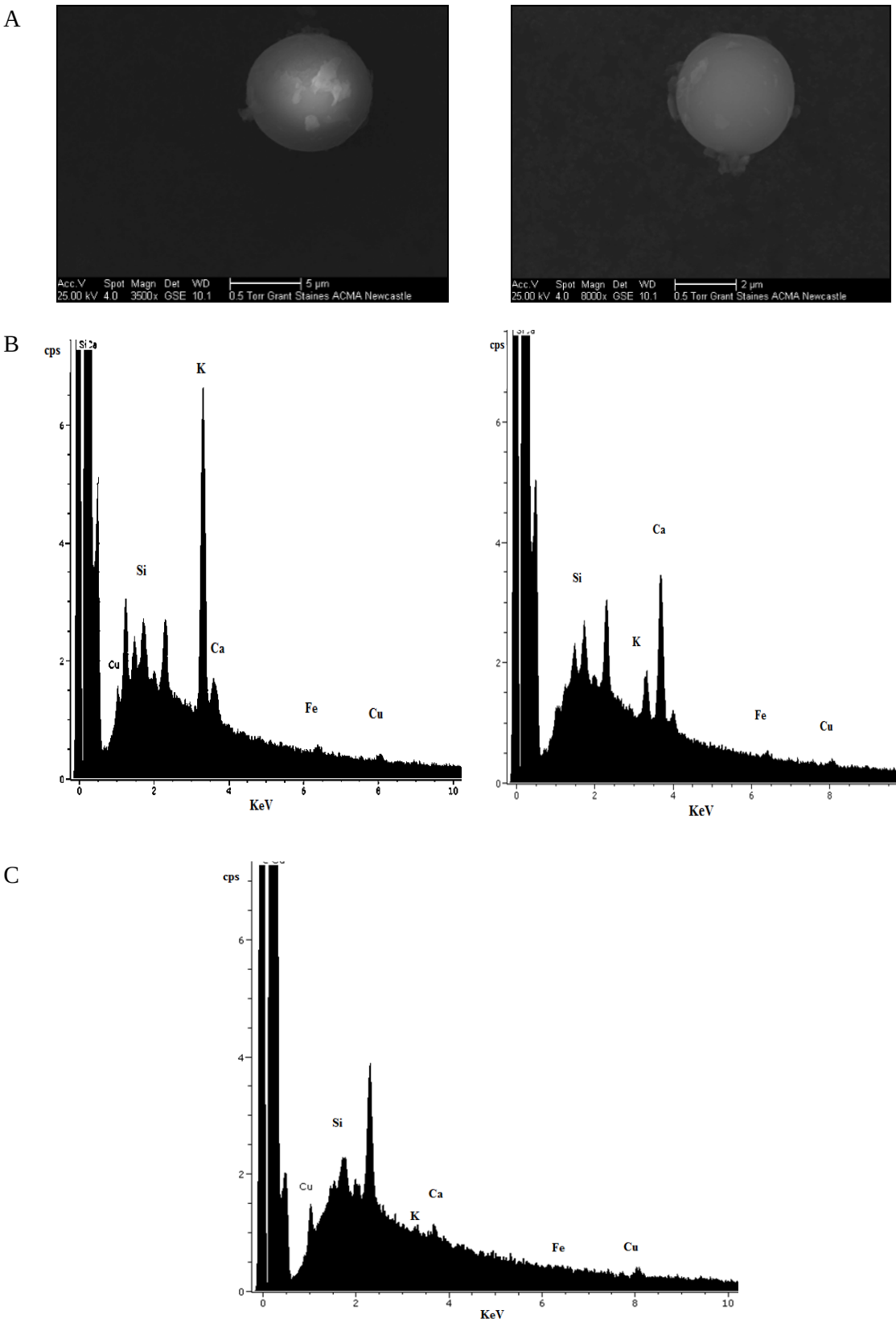


Figure 3.5. (A): Electron micrographs and (B): Energy dispersive x-ray analysis (EDAX) of two individual alcohol extracted urate spheres from chickens on the metal contaminated allotment (collected August 2005). (C): EDAX of the carbon stub representing the non-sphere background analysis.

The background analysis of the carbon stub (Fig. 3.5C) had several contaminants especially sulphur (2.4KeV) from an adhesive material (M. Staines, personal communication). Although Cu appeared to be detected by EDAX in these spheres the concentration was no greater than the background analysis so this was discounted. If Pb or Zn had been detectable they would have appeared as peaks at 2.5 and 8.5KeV respectively on the EDAX analysis.

3.4. Discussion

3.4.1. Metal concentrations and properties of contaminated soil

The lead concentration in the pre-remediation chicken pen soil (Table 3.1) was above the soil guideline values of 450mg Pb kg^{-1} dry mass, confirming this allotment's contaminated land status (DEFRA, 2000). Also Cu and Zn exceeded the now withdrawn Inter-departmental Committee on the Redevelopment of Contaminated Land (ICRCL) intervention concentrations of 130 and 300mg kg^{-1} dry mass respectively. These elevated concentrations of Pb, Cu and Zn in the soil samples, although characteristic of the incinerator bottom ash added to the site (reported as 760, 870 and 1100 mg kg^{-1} dry mass respectively, Pless-Mulloli et al., 2004), may also have originated from other sources. These include the use of agro-chemicals on the gardens (Rimmer et al., 2006) and burning rubbish especially plastics (Meharg and French, 1995).
Chicken manure is likely to be responsible for the chicken pen SOM values being higher than the 7-13% reported for allotment garden soils (Clark et al., 2006). This was backed up by the later analysis of chicken guano using the same technique giving an organic matter content of 76%. Although the soil metal load may also be derived from chicken manure input, due to high Cu and Zn inclusion in commercial diets (Mohanna and Nys, 1998), I showed on analysis that the feed of these birds was low in these metals, discounting this as a major source of soil metal contamination. From my findings the combination of neutral pH and high SOM of these samples would be expected to reduce metal bioavailability in the contaminated chicken pen soil (Clark et al., 2006).

3.4.2. Metal concentrations in chicken feed

Chickens on the control allotment were fed rations high in added Cu and Zn with concentrations being comparable to literature values (Nicholson et al., 1999). However birds on the contaminated site before and after remediation were fed a ration significantly lower in Cu and Zn. As the control birds had no access to soil, the feed was the major source of metal uptake, in contrast to the birds on the contaminated site in which soil was the major metal exposure route. The Pb concentrations in both rations were low and would not be expected to significantly contribute to the uptake of this metal by chickens. A consequence of the higher concentrations of Cu and Zn in the feed of control site chickens compared to the contaminated soil exposed birds prevented a meaningful comparison of these metals in biomonitor samples. However, post-remediation when the soil contribution to uptake of these metals was drastically reduced in the birds on the contaminated site, the different feed concentrations (Table 3.2) were reflected in the guano samples (Fig.3.5).

3.4.3. Biomonitor samples

Metal concentrations in eggs

In the current study (bearing in mind the LOD of 2mg kg^{-1}) egg yolk and shell were unsuitable materials to biomonitor increased Pb exposure from the contaminated soil, confirming that lead has a low transfer to eggs (Walsh, 1990). However the sensitivity of the analysis method used here may have compromised my results and should not preclude eggs from being a valid material for biomonitoring. Pb concentrations in egg yolks and shells from Pb exposed chickens (from ingested Pb-based paint chips in their environment) were 0.4 and 0.45mg kg^{-1} respectively and significantly above concentrations in eggs from control birds (Trampel et al., 2003). In addition Mazliah et al., (1989) reported eggshells from Pb dosed hens had 6-12 times the Pb concentration of eggshells from controls; while the Pb content of the egg yolks from dosed hens was significantly higher than controls. In a study on environmental uptake of heavy metals by house sparrows (*Passer domesticus*) there was significant correlation between Pb, Cu and Zn in egg shell and egg content (Swaileh and Sansur, 2006), making the shell a valuable biomonitoring matrix for these metals. However Grand et al., (2002) cast doubt on the value of eggs for biomonitoring Pb exposure in birds, reporting no correlation between blood and egg Pb concentrations in two species of wild duck.

Skrivan et al.,(2006) raised the dietary Cu intake of laying chickens increasing Cu concentrations in egg yolk and shell from 3.5 to 5.0mg kg^{-1} and 2.0 to 2.5mg kg^{-1} (dry mass) respectively. However these small increases required more than a ten-fold elevation in dietary Cu intake, further emphasising that eggs are an insensitive monitor for this metal. In a study on concentrations of heavy metals in laying great tits (*Parus major*) and their eggs, Dauwe et al.,(2005) hypothesised that egg content or shell were unsuitable as a measure of exposure because Cu and Zn are under physiological control. These limitations on eggs as biomonitors for Cu and Zn exposure are reflected in egg yolks in this study but the egg shell values are too few to draw any conclusion. In the light of these findings no eggs were collected from the chickens after remediation for metal analysis.

As the eggs from the contaminated allotment were used for human consumption an estimate of dietary Pb exposure was calculated in a manner similar to Trampel et al., (2003). By assuming Pb concentration in the egg yolks, typically 8-9g dry mass, had reached the LOD (2 mg kg^{-1} dry mass), a 60kg person would require a daily intake of greater than 12 eggs, to exceed the provisional tolerable weekly intake (PTWI) of 0.025mg kg^{-1} body mass (JFWEC 1999).

Metal concentrations in feathers

While feathers reflected the elevated Pb exposure on the contaminated allotment compared with the control site, Zn concentrations were no different and Cu concentrations were significantly lower in feathers collected from the contaminated site compared to those from the control. Again the results obtained in relation to essential metals Cu and Zn could be explained by their homeostatic regulation in birds (Dauwe et al., 2003) and it is known that the internal deposition of heavy metals in feathers is only a fraction of the total body burden, with the exception of mercury (Veerle et al., 2004) and organo-tin (Kannan and Falandysz, 1997). The surface affinity of feathers to bind heavy metals is shown by their use in wastewater clean up (Al-Asheh et al., 2003) and how cleaning techniques can add to their metal content (Hogstad et al., 2003). Pb is recognised as principally a surface contaminant in feathers (Nam et al., 2004), so the higher concentration of Pb we report here may be from contaminated soil or guano accumulation on their surface. However similar differences from surface contamination with soil derived Cu and Zn could be masked by feed or guano contamination from their typically high concentrations in commercial chicken feed (Mohanna and Nys, 1998). As the inter-moult period dictates how long the feathers have to accumulate surface metals

(Veerle et al., 2004) and this may not be the same on the two allotments, this factor could also adversely influence the results.

The reported lack of correlation between Pb concentrations in different feather groups or between feather and blood Pb concentrations in blackbirds (*Turdus merula*) from Pb polluted urban areas (Scheifler et al., 2006), further confirms the limitations of this technique for measuring bioavailable Pb exposure.

Feathers were not collected following remediation as it was considered likely that after just 6 months they may still reflect pre-remediation contamination concentrations. This was because typically adult domestic chickens moult only once a year in autumn (King and McLelland, 1975) and the soil remediation took place in early November 2006. As a result post-remediation feathers were likely to have been formed prior to remediation, so both internally and surface accumulated metals in the feather could be derived from contaminated soil exposure.

Metal concentrations in whole guano

Pb concentrations in whole guano from birds on the contaminated allotment were elevated over control samples (Fig.3.5.) and normal background concentrations reported in the literature (Nicholson et al., 1999). This indicates that oral uptake of contaminated soil was responsible for the high Pb guano concentrations and suggests whole guano could be a suitable biomonitor for Pb exposure. By assuming the majority of ingested Pb was from the contaminated soil and dietary Pb is concentrated 3.25 times in chicken guano as reported for Cu (Kunkle et al., 1981), the calculated percentage soil uptake on a dry matter basis was 8%. This is in agreement with estimates of soil intake by chickens reported in the literature (Beyer et al., 1994).

Following remediation of the contaminated allotment with clean soil (having a mean value of 59 mg Pb kg^{-1}dry mass), the whole guano lead concentration dropped to a median of 31 mg Pb kg^{-1}. But if the same soil intake value of 8% as determined above is assumed, on calculation this should have resulted in a guano concentration of 15.6 mg Pb kg^{-1}, which is nearer to the value of 8.3$\pm$5.0 mg Pb kg^{-1}obtained from the guano of chickens on the control allotment (Fig. 3.5.). This elevated concentration of Pb in guano after site remediation is likely to have resulted from the urine component of the guano (see *3.4.3.4.* below).

Cu and Zn concentrations in the guano of chickens on contaminated soil were not apparently different from guano metal concentrations sampled from the control site (Fig.3.5.), while guano samples post remediation appeared to have lower metal

concentrations than those found in guano from control and pre-remediation birds. This result shows how the typically high dietary inclusion rates of these metals (Mohanna and Nys, 1998) in the ration fed to the control site birds, masked the elevated uptake from the soil in birds on the contaminated allotment. The similar metal concentrations found in the guano samples would suggest birds from the control and contaminated sites are being equally exposed to Cu and Zn. This is not the case because the metals come from different sources (either food or contaminated soil) and so may be in different chemical forms, which can affect their relative bioavailability (Ruby, 2004). Cu and Zn in guano from the control allotment birds is entirely from the feed, and these metals are reported to be poorly absorbed in chickens with less than 6% being retained in the body from commercial diets (Mohanna and Nys 1998). As concentrations of Cu and Zn are low in the feed (5.7 and 20mg kg^{-1}) but high in the soil (273 and 800mg kg^{-1}) on the contaminated allotment, it can be calculated that most of the guano derived metal originates from the soil intake of these chickens. Assuming again a soil intake of 8% (dry mass basis), for each kg of dry diet consumed the

Zn intake from soil would be 8% of 827 i.e. 66.2mg Zn, while from feed 92% of 20 results in an intake of 18.4mg Zn; similarly the Cu intake from soil is 8% of 273 i.e. 22mg Cu compared with the lower intake from the feed being 92% of 5.7 i.e. 5mg Cu. The calculated intake of Cu and Zn in the control chickens (being entirely from feed) of 10.6mg Cu kg^{-1} and 60.5mg Zn kg^{-1} was lower than the intake of chickens on the contaminated allotment, 27mg Cu kg^{-1} and 84.6mg Zn kg^{-1} respectively. As this difference between contaminated and control chickens is not shown in the whole guano analysis (Fig. 3), it may suggest the soil derived metals are more readily absorbed from the digestive system as a consequence of being more bioavailable. In a separate study I determined the Cu and Zn bioavailability in the soil from the contaminated allotment using an *in vitro* method (Rieuwerts et al., 2000) and found them to be high (75-84%). This is consistent with these metals' likely origin from added anthropogenic products of combustion (incinerator bottom ash) in contrast to geological sources found in background soils (Rieuwerts et al., 2000).

This highlights a potential problem of using whole guano in metal exposure studies because it does not take into account variations in metal bioavailability (Ruby, 2004).

AUS

Extraction

Because the method of extraction depends on the formation of a persistent suspension of AUS, drying and grinding the guano samples prior to extraction was not carried out, as this would have increased the formation of similar fine particulates. Although small Pb-rich particulates (<37μm) have been reported in urban soils (Clark et al., 2006) and such high density particulates are difficult to separate gravitationally (Mercier et al., 2001) no serious contamination problems were encountered. This was evidenced by microscopic examination of the AUS samples (Fig.2.1A).

The post-remediation extracted AUS (Fig.3.6.) appear to show urine to be a route for Pb excretion. Additionally the persistent high AUS concentration, despite low feed and soil values, confirms this metal's presence in extracted urate spheres is not simply from faecal contamination.

Metal concentrations

In contrast to other methods (eggs, feathers or whole guano), AUS samples from chickens on the contaminated soil, when compared with controls, appeared to give a better representation of the birds' elevated exposure to all three heavy metals (Fig.3.6.). This may be a consequence of the AUS content consisting of heavy metals entirely derived from the bloodstream following digestive absorption, hence representing the fraction of metals from environmental sources that are bioavailable to the birds. In comparison AUS samples from the chickens 6 months after site remediation, reflected the reduced Cu and Zn exposure from the clean replacement soil. Interestingly Pb concentrations in the AUS remained high after site remediation. This continued elevated excretion may be a consequence of bone mobilized for egg production (Dacke, 2000; King and McLelland, 1975), releasing chronically sequestered Pb deposits into the bloodstream. Bone Pb concentrations in birds account for approximately 90% of the body burden, with egg laying females accumulating more than males (Scheuhammer et al., 1999). It would be expected following remediation that urine Pb concentrations should decline as the bone Pb is excreted over time. In humans this decline may be quite prolonged (decades) and varies with bone type, metabolic state, and subject age (Hu et al., 1998). Similarly, whether or not a chicken was laying eggs, would be expected to affect the rate of bone mobilization and therefore Pb urinary excretion. Pain et al., (1997) reported that blood Pb concentrations remained elevated for longer (several months) following higher exposure from Pb shot ingestion in marsh harriers (*Circus aeruginosus*). Persistent excretion of this quantity of Pb 6 months after reducing the

birds' Pb intake, may suggest substantial bone deposits of Pb from their previous prolonged exposure.

Cu and Zn do not substantially accumulate in the body like Pb (Walsh, 1990), being essential metals under metabolic control. Therefore they did not show a prolonged excretion in the AUS following remediation. AUS concentrations of Cu and Zn are excess to the bird's requirement excreted under homeostatic control, while Pb AUS concentrations reflect unregulated blood concentrations. In the light of blood concentrations of essential metals being kept within a normal range, AUS sampling may be a better measure of excessive exposure than blood, casting doubt on blood being the 'gold standard' for monitoring purposes (Furness, 1993). For the nonessential metal, Pb, AUS concentrations could be expected to reflect blood concentrations. However the present study showed AUS concentrations may not directly relate to the birds' current intake due to Pb accumulation in bone and its subsequent release due to bone remobilisation.

EDAX analysis

The EDAX analysis of AUS has been reported previously by Casotti and Braun, (1997 and 2004), where they determined the ionic composition of individual urate spheres. Chicken urate spheres were reported to contain Mg using EDAX analysis (Casotti & Braun, 1997). However in a later paper the authors suspected it had been from background analysis of the stub (Casotti & Braun, 2004).

In the present study the EDAX analysis of individual AUS was unable to detect any of the three metal ions identified with the contaminated soil. This was because the sensitivity of the EDAX analysis is restricted to 0.1% (dry mass) of a sample, equivalent to 1g/kg and several times higher than the concentrations detected in the AUSby ICP-OES.

The elemental analysis by EDAX of individual AUS (Fig.3.7B) showed K and Ca were the predominant cations, in agreement with Casotti & Braun, (2004). However the relative concentrations of K and Ca varied substantially between the two spheres. The theory that urate spheres from laying birds have elevated Ca concentrations (over K) may however still be correct because this analysis was from pooled guano samples and not all the birds were laying eggs at the time.

3.4.4. Health implications for chickens ingesting heavy metal contaminated soil

The toxic and sub-lethal effects of ingested Pb, Cu and Zn on birds varies widely between species, age, sex and the chemical form of each metal (Eisler, 2000 and refs within). Experimental poisoning of captive birds with Pb showed wide species variation in susceptibility (Beyer et al., 1988), with a similar finding reported for Cu and Zn (Eisler, 2000 and refs within). Among avian species domestic chickens are comparatively resistant to Pb toxicosis, with a diet containing 1.85g Kg^{-1} as Pb acetate given over 4 weeks to domestic cockerels being non-lethal (Franson and Custer, 1982). Diets in domestic chickens with concentrations above 500 and 2000 mg Kg^{-1} (dry mass) Cu and Zn respectively are reported to be toxic (Eisler, 2000 and refs within). Such a relative insensitivity of the domestic chicken to metal toxicosis compared to other avian species (Eisler, 2000) combined with its habit of ingesting soil, makes it a suitable sentinel species to biomonitor heavy metal contaminated soils.

By assuming 8% of the chicken diet on the contaminated allotment consisted of soil and that from analysis (3.3.1.) it had maximum Pb, Cu and Zn concentrations of 680, 210 and 860 mg Kg^{-1} (dry mass) respectively, the soil contribution to metal intake can be calculated. The remaining 92% of the diet represents the feed given to the birds, which had maximum concentrations of Pb, Cu and Zn of 1.7, 6.6 and 23.7 mg Kg^{-1} (dry mass) respectively. From these values the maximum metal concentrations in the combined diet of soil and feed can be calculated (Table 3.6), being 55.6, 23.1 and 90.8 mg Kg^{-1} (dry mass) for Pb, Cu and Zn respectively. These estimates for metal intake are all well below the reported toxic concentrations for domestic chickens and would explain why no signs of metal toxicosis (Eisler, 2000) were reported in the birds on the contaminated allotment.

Table. 3.6. Dietary intake of lead (Pb), copper (Cu) and zinc (Zn) from the combined soil and feed components, in chickens on the contaminated allotment. All values in mg Kg^{-1} (dry mass).

	Soil component		Feed component		Total dietary concentration this study	Toxic concentration reported*
Metal	Max value	8%	Max value	92%		
Pb	680	54	1.7	1.6	55.6	>1,850
Cu	210	17	6.6	6.1	23.1	>500
Zn	860	69	23.7	21.8	90.8	>2,000

* Eisler, (2000).

3.5. Conclusions

In the context of heavy metal pollution, avian biomonitoring attempts to determine a bird's internal exposure to bioavailable metals from the environment, classically represented by circulating blood concentrations (Furness, 1993). It is evident that current non-destructive biomonitoring methods using eggs, feathers or guano may not adequately reflect this. Egg and feather production draw upon both current intake and sequestered body reserves, so may not reflect current heavy metal body uptake from environmental exposure. Also, the homeostatic control of essential metals (e.g. Cu and Zn) in blood restricts their deposition in eggs and feathers to within a normal range (Walsh, 1990). Feathers may gain variable amounts of surface accumulated heavy metals, which on analysis are indistinguishable from bioavailable internal deposits (Scheifler et al., 2006). Analysis of guano is complicated by its being a mixture of faecal and urinary excretions. The faecal heavy metals may have varied bioavailability, with non- bioavailable metals simply transiting the digestive system.

The present study has shown that metals can be measured in AUS but not that concentrations reflect biological availability because there was no assessment of availability or uptake. This could have been achieved by measuring metal residues in tissues and/or blood from the birds. The short comings of the study include the lack of a proper control group: the control birds in this study were at a different site, were not kept on uncontaminated soil, and their diet was different. Taking representative samples of bulked guano and AUS meant that statistical analysis was not possible for these samples, giving only a measure of technical rather than biological variation. Guano should have been collected and analysed from individual birds, and concurrently residues of metals determined in their blood a range of tissues, to assess uptake. Another problem with the study was that metal concentrations in pre- and post-remediation samples were measured using different methods carried out at different laboratories. This seriously affects any comparison between the two measurements and ads to the problem of using bulked samples.

Chapter 4.

An investigation into heavy metal concentrations in breeding seabirds

4.1. Introduction

An earlier unpublished study carried out at Newcastle University (R.M. Bevan and I. Singleton, personal communication), reported high metal concentrations in a species of marine fish collected in 2003 from the North Sea (Table 4.1). The fish was a small benthopelagic species called the lesser sandeel (*Ammodytes marinus*), hereafter sandeel, caught in the vicinity of the Farne Islands (55°38'N; 1°37'W) off the Northumberland coast in the UK.

Table 4.1. Copper (Cu), lead (Pb), zinc (Zn) and cadmium (Cd) concentrations (mg kg^{-1} dry mass) recorded in pooled samples of whole lesser sandeels (*Ammodytes marinus*) caught in 2003 from one inshore (Ross Bank) and two offshore (Inner Farne and Longstone Banks) sites in the vicinity of the Farne Islands. For comparison published sandeel data and sea bed metal concentrations are included.

Trawl site	Date caught	Cu	Pb	Zn	Cd
Ross Bank	17/06/03	109.2	204.5	365.6	44.8
Ross Bank	24/07/03	83.0	97.0	297.5	0.0
Inner Farne Bank	17/06/03	162.4	179.1	738.8	15.7
Inner Farne Bank	24/07/03	99.3	95.0	166.9	8.5
Longstone Bank	17/06/03	103.0	135.1	159.7	29.4
Longstone Bank	24/07/03	86.0	165.0	151.9	41.8
Isle of May (CEH, 2003/04)*	2004	-	-	152	-
Fish meal (Moren et al.,2006)**		4	0.09	80	0.19
Sediment from Ross Bank†		<3.0	16.0	140.0	<0.6

*Mean Zn concentration (n=17) in sandeels from a CEH study.
** Typical analysis of fish meal from North Sea fish species 30% sandeels composition.
†Analysis of sea bed sediment from the Ross Bank trawl site (R.M. Bevan and I. Singleton, personal communication).

This finding would have major ramifications for the transfer of heavy metals in the marine environment of the Farne Islands. Not least from the pre-eminence of this fish in North Sea food chains as the major prey of 15 fish species and numerous seabirds and mammals in the region (Furness, 2002). There are also economic consequences from such a metal contamination because sandeels typically constitute the largest single species fishery in the region; illustrated by this species being 37% by mass of the total North Sea fish caught in 1995 (OSPAR, 2000). Additionally such elevated heavy metal concentrations may be relevant to recent reports of declining seabird populations and falling sandeel stocks in the region (Mitchell et al., 2004; Mavor et al., 2006). Seabirds are recognised sentinels of environmental change, which typically involves them being

impacted from altered food supplies (Croxall et al., 2002). For this reason a study was devised to investigate whether these high metal concentrations in the sandeels were impacting on the Farne Island seabirds. This was enabled by the fact that tissue concentrations of heavy metal contaminants from marine birds are widely used to biomonitor heavy metal environmental pollution (Burger and Gochfeld, 2000 and 2003). This study was also an opportunity to further investigate the utility of extracted avian urate spheres (AUS) for biomonitoring. This was achieved by comparing heavy metal concentrations in AUS from the guano with tissue samples (liver, bone, feather, eggshell and whole chicks) from seabirds in the colony.

The Farne Islands constitute a major summer breeding site for many seabirds. Three species which almost exclusively catch sandeels to feed their young are Atlantic puffins (*Fratercula arctica*), black legged kittiwakes (*Rissa tridactyla*) and Arctic terns (*Sterna paradisaea*), hereafter referred to as puffins, kittiwakes and terns respectively. For this reason samples were collected from colonies of these birds on Brownsman Island in the Farne Island archipelago off the Northumberland coast. Samples from terns predominated because their nests were more accessible, in contrast to the underground burrows of puffins and cliff ledge sites of kittiwake nests.

Seabirds have been widely used to biomonitor marine pollution, including metals, using various destructive and non-destructive methods (Furness and Camphuysen, 1997; Gochfeld, 1997). Because many seabird chicks and fledglings die every year from natural causes, usually starvation, their collection provided tissue samples usually only available through destructive sampling. In addition non-destructive samples were collected, consisting of fresh guano and discarded egg shells from hatched chicks.

As the initial results from both tissue and urine samples showed no significant heavy metal transfer to the birds, freezer-stored sandeels caught in the same region between 2002 and 2006 were analysed to check the earlier findings.

The overall aim of the work in this Chapter was to measure AUS metal concentrations to signify the seabirds' exposure to elevated levels in their sandeel dietin combination with analysis of tissue samples to enable comparison with reported values for such an exposure. The objective was to provide evidence that AUS could be used as a medium for biomonitoring environmental heavy metal contamination.

4.2. Materials and methods

4.2.1 Background

To measure the metal concentrations in seabird diets, sandeels caught by trawler in the vicinity of the Farne Islands and uneaten fish from around the tern nest sites were analysed. The uneaten fish consisted of one species, the snake pipefish (*Entelurus aequoreus*), later referred to as pipefish. Since 2003 this fish species has increased dramatically in abundance off the Farne Islands and in other northern European sea areas (Harris et al., 2007). As a result, pipefish, along with sandeels, have become the two prey species predominantly fed to the seabird chicks (R.M. Bevan, personal communication).

4.2.2 Sample collection and storage

Samples from the Farne Islands were mostly collected by Newcastle University School of Biology undergraduate students, under the supervision of Dr R. Bevan, while taking part in project work. The fresh guano samples were collected in absolute alcohol as no freezer was available on the island. Consequently whole guano was not analysed, only the extracted AUS following the procedure described in Chapter 3. It was assumed that guano samples from kittiwakes were a mixture of both adult and chick in origin, being collected from the rocks below each nest. The tern and puffin guano samples were collected off rocks and vegetation on the island. As the chicks of both species are nest-bound, these represented samples from adult birds.

Samples of pipefish, chicks, fledgling tissues, egg shells and guano were mostly collected in June and July 2006 from Brownsman Island. Of these the tern chicks and fledgling livers (removed on site) and the fresh guano samples were collected and stored immersed with GPR absolute ethanol in 50mL polypropylene centrifuge tubes (Fisherbrand®). The tern fledglings, used for bone and feather samples, having little remaining soft tissue (fly-eaten), were collected dry and frozen later for storage. Similarly, egg shell and pipefish samples being dry were frozen later for storage. The fewer samples collected from the same site in June and July 2004 consisted of two whole chicks and six fledglings (used for liver analysis), and were all stored frozen. The sandeel samples had been freezer-stored at -20^{0}C following trawls from three catch locations around the Farne Islands carried out by the Marine Biology Unit, these being

Ross Bank, Inner Farne Bank and Longstone Bank. Of these Ross Bank constituted an inshore trawl site within one nautical mile of the mainland while the other two were offshore sites.

4.2.3 Sample preparation

The various samples other than the sandeels were prepared as described below, then oven-dried to constant mass at 60^0C. No determination of % dry mass was carried out because most samples were in a semi-dehydrated state when collected, or saturated with alcohol.

A mixture of breast and wing feathers were plucked from each of six fledgling tern carcases, washed in warm tap water and rinsed in 18MΩ deionised water to remove loosely adherent external contamination.

Both left and right wing bones consisting of humerus, radius and ulna, were dissected from each fledgling tern carcase, with all soft tissue scraped off with a stainless steel knife.

Egg shells, after stripping out the internal membranes along with any pre-hatch guano, were washed in warm tap water and rinsed with 18MΩ deionised water.

Solid urine was extracted from each sample of adult and pre-hatch guano using GPR ethanol as described earlier in Chapter 3.

The tern chicks and fledgling liver samples from 2006 were drained of excess ethanol, while the equivalent 2004 frozen samples were defrosted overnight prior to both sets being oven dried.

Following oven drying, all the samples were individually homogenised by grinding then sealed in 50mL polypropylene centrifuge tubes (Fisherbrand®) and dispatched for metal analysis by a UKAS accredited laboratory. The measurement of metal concentrations in putatively whole homogenised chick samples was to determine the whole body burden of metals. This was similar to a method reported by Van den Steen et al., (2009) to measure the total body burden of organic halogenated pollutants in blue tits (*Parus caeruleus*).

The sandeel samples were defrosted at room temperature prior to freeze-drying over a 24-hour cycle in individual plastic weighing boats. The total dry mass of each fish was accurately recorded to 0.01grams using a four-point balance. In most cases the entire

fish was processed, while an accurately weighed 1 gram aliquot of a well-mixed homogenised ground sample was used of fish weighing more than 1.5g.
Digestion of each dry sandeel sample was carried out in Kjeldahl tubes using freshly prepared aqua regia (a 1:3 mixture of concentrated nitric and hydrochloric acids) heated at 95^{O}C in a Gerhardt heat block for 30 min. After being left to cool to room temperature, each digested sample was transferred to a 25mL volumetric flask made up to volume with double distilled 18MΩ water and thoroughly mixed. From each sample a 5mL aliquot was filtered (Puradisc® 25AS 0.45µ syringe filter Whatman) into individual polypropylene centrifuge tubes (Fisherbrand®) prior to analysis by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

4.2.4 Sample analysis

Cd, Pb, Cu, Zn, Hg and Sn concentrations for all samples other than the sandeels, were determined by a UKAS (United Kingdom Accreditation Service) accredited laboratory (www.aes-labs.co.uk). The in-house method of this laboratory used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after a 2 hour acid reflux pre-digestion. Levels of detection (mg kg^{-1} dry mass) were poorer for some of the smaller samples (<0.5g) but were generally >0.06 for Hg, >0.3 for Cd and Sn, >2 for Cu and Pb and >4 for Zn. Although Hg and Sn were not included in the earlier metal analysis of the 2003 sandeels, they were included as both Hg (Walsh, 1990; Monteiro and Furness 1995) and organic Sn compounds (Walsh, 1990; Kannan and Falandysz, 1997; Tanabe et al., 1998) are implicated in adverse ecotoxicological effects in marine biota.
Metal analysis of the filtered acid digested sandeel samples was performed using a Unicam 701 ICP-OES (Unicam Instruments, Cambridge, England). Calibration standards (Sigma) were freshly made up for each metal, having limits of detection in a complicated matrix solution of 0.1mg L^{-1} for Pb and 0.01 mg L^{-1} for Cd, Cu and Zn. The contribution of metal concentrations in the two acids used for digestion was determined by running acid blanks without samples, this value being subtracted from the sample results before calculating metal values. The resulting limits of detection in dry fish samples were Pb 2.5 mg kg^{-1} and 0.25 mg kg^{-1} for Cd, Cu and Zn. All metal concentrations in this report refer to dry mass samples only.

4.2.6 Data analysis

The Minitab® (version 15) programme was used for statistical analyses and graphical output. Statistical analysis of the AUS samples could not be carried out because they were derived from pooled guano samples so would only reflect technical variability. The small number of tissue samples, combined with numerous metal values below the detection concentration, meant that the data were analysed using non-parametric statistics. These data were presented as median and range of metal concentrations in mg kg^{-1} on a dry mass basis with the Mann-Whitney U test used to determine significant differences between data sets at a 95% confidence interval. The larger number of sandeel samples allowed for parametric analysis of their metal concentrations after confirming normality using the Shapiro-Wilk W goodness of fit test and equal variance using Levene's test. Regression analysis was used to determine correlations between fish dry mass and metal concentrations. The parametric two-sample t-test was also carried out on the sandeel data to determine significant differences in metal concentrations between two trawl sites and two fish sizes. Furthermore ANOVA followed by post-hoc analysis was used to analyse the influence of year on sandeel metal concentrations . P values less than 0.05 at a 95% confidence interval were taken to show a statistically significant difference.

4.3. Results

As none of the samples had detectable concentrations of Sn this metal was excluded from the results.

4.3.1 Fish samples

The pipefish (n=5) had below detection concentrations of all metals except Cu and Zn for which the median (and range) values were 2 (1-3) mg kg^{-1} and 50 (40-86) mg kg^{-1} respectively.

Although 60 individual samples of sandeels were processed and analysed as described, over half were lost from a combination of a faulty thermostat on the heat block used for sample digestion and contaminated Kjeldahl digestion tubes. As a result only 22 sandeel samples out of the original 60 are reported on in this study.

The three variables; total dry mass, year of collection and trawl site were considered to be relevant in terms of the sandeel metal concentrations. As only 2 fish were analysed from the Longstone Bank trawl, a comparison between trawl sites was only carried out between the other two sites.

To determine if a relationship existed between total dry mass and metal concentrations, simple scatter plots were produced, which in Cd, Pb and Zn suggested a curvilinear relationship. For this reason logarithmic transformed total dry mass values were used to show graphically how the age of the sandeels, as measured by their total dry mass, influenced metal concentrations (Fig.4.1.). Significant negative correlation was shown between the log dry mass of the fish and three of the metals. Specifically Cd: R^2= 77.6%, P< 0.001, Pb: R^2= 39.1%, P= 0.002 and Zn: R^2= 39.8%, P= 0.002. However no such correlation was shown for Cu concentrations, having values of R^2= 1.5% and P= 0.582.

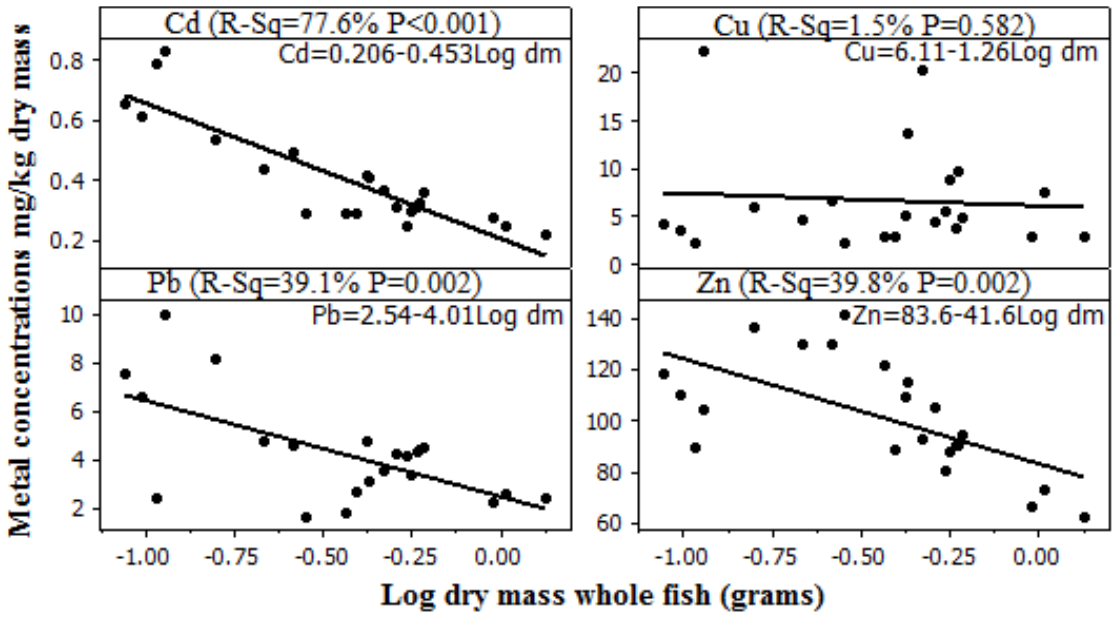


Figure 4.1. Scatter plots showing how cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) metal concentrations vary with the log transformed total dry mass of whole sandeels (*Ammodytes marinus*) caught in the Farne Island sea area.

As Harris et al. (2008) reported significant differences in oil concentrations between size 0 (<0.15g dry mass) and older sandeels, the fish mass were divided into similar size groups of ≤0.15g and >0.15g. Size 0 fish had significantly higher Cd and Pb concentrations than older fish (Student t-tests: Cd: t = 7.62, df = 20, p<0.001; Pb: t = 2.82, df = 20, p = 0.011), but there was no significant difference for Cu and Zn

concentrations between the two size categories (Student t-tests: Cu: t = 0.56, df = 20, p= 0.585; Zn: t = 0.37, df = 20, p= 0.717).

Figure 4.2 compares metal concentrations in sandeels between the Ross Bank and Inner Farne Bank trawl sites. Fish from the coastal trawl site of Ross Bank, had significantly higher concentrations of Cd and Zn than fish from the more off shore Inner Farne Bank trawl site (Student t-tests: Cd: t= 3.28, df = 19, p= 0.004; Zn: t= 3.45, df = 19, p= 0.003), while Cu and Pb concentrations were not significantly different (Student t-tests: Cu: t = 0.72, df = 19, p= 0.479; Pb: t= 1.54, df= 19, p= 0.140).

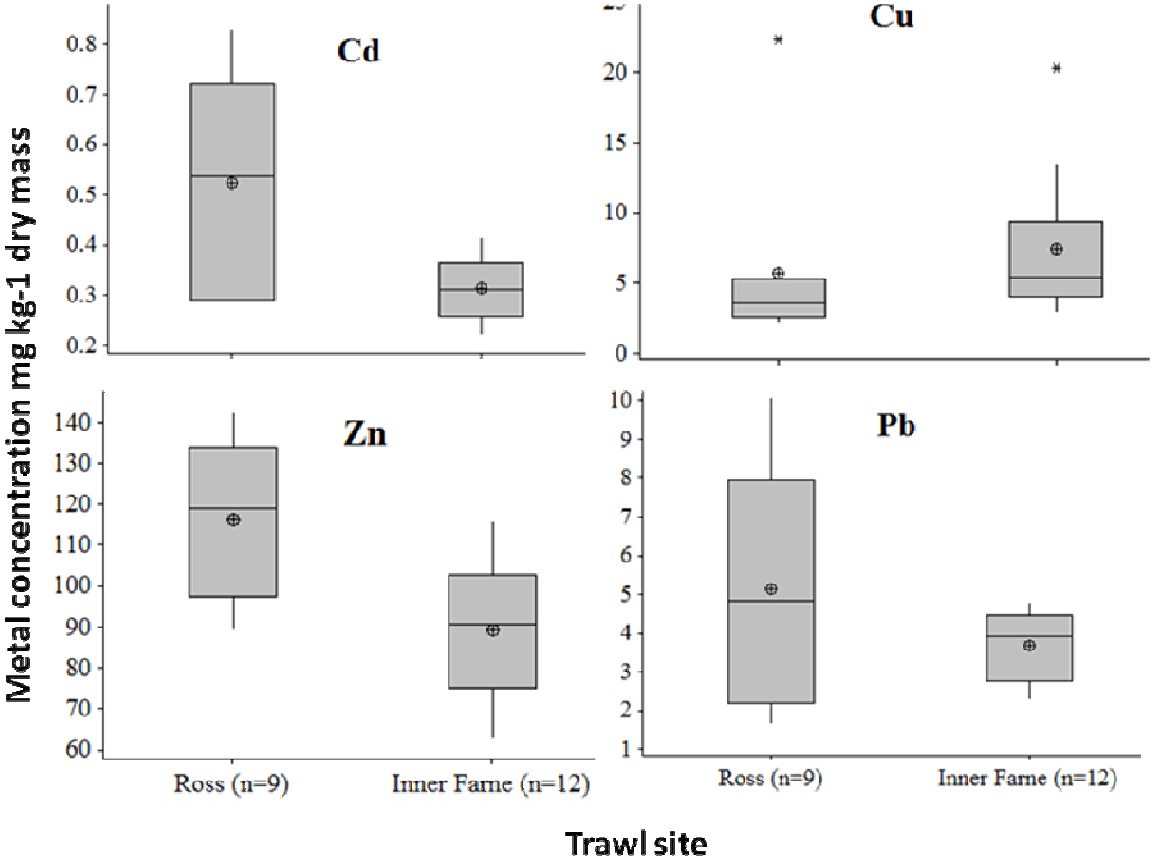


Figure 4.2. Box plots comparing cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) metal concentrations in sandeels (*Ammodytes marinus*) between the Ross Bank (inshore) and Inner Farne Bank (offshore) trawl sites. Each box represents the interquartile range of metal concentrations around the median value, whiskers denote maximum and minimum values and an asterisk any outlier. The crossed circle signifies the mean values for each trawl site.

When comparing metal concentrations between trawl sites (Fig.4.2.) it is important to take into account that the Inner Farne fish were significantly larger (Fig. 4.3.) than the Ross fish (Student t-test: t= 4.61, df = 19, p<0.001) and because smaller fish had higher Cd, Pb and Zn metal concentrations (Fig.4.1.), their size, rather than location, may be

influencing these metal concentrations. To rule this out, similar-sized fish from each site should be compared. However, too few samples were available to undertake this analysis.

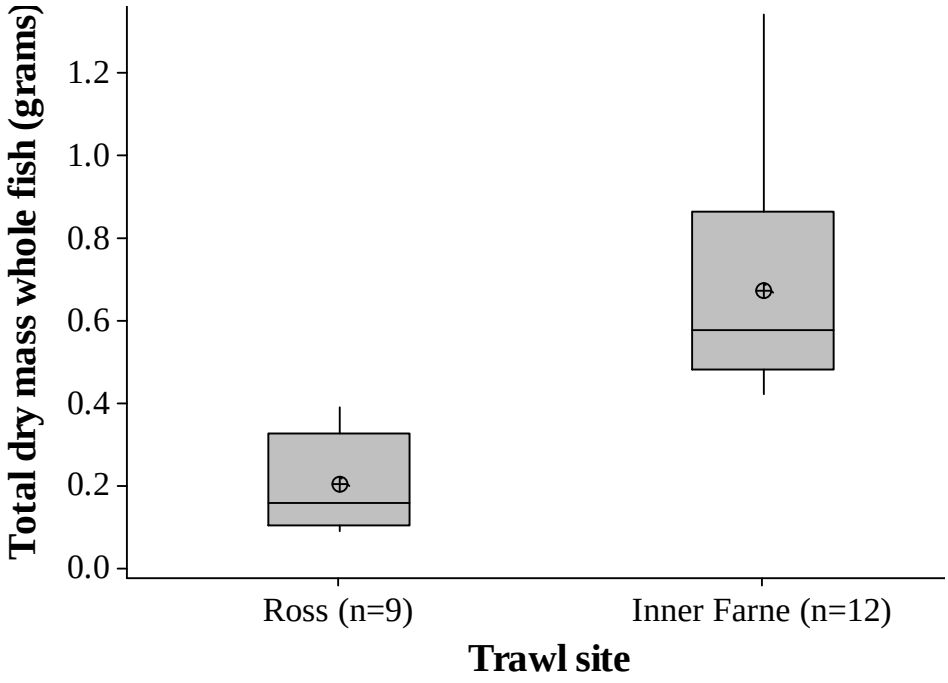


Figure 4.3. Box plots comparing total dry mass of sandeels (*Ammodytes marinus*) between the Ross Bank (inshore) and Inner Farne Bank (offshore) trawl sites. Each box represents the interquartile range of total dry mass around the median value; whiskers denote maximum and minimum values. The crossed circle signifies the mean values for each trawl site.

A one-way ANOVA of metal concentrations between fish caught in different years showed Cd concentrations differed significantly ($F(4,17) = 3.981$, $p = 0.019$). However Cu, Pb and Zn concentrations were not significantly different between fish caught in different years. Tukey post-hoc comparisons of the five years indicate that the 2003 caught fish ($M = 0.56$, 95% CI [0.40, 0.72]) had significantly higher Cd concentrations than the 2004 caught fish ($M = 0.32$, 95% CI [0.20, 0.43]), $p = .033$. Paired comparisons between the 2003 caught fish and the other years were not significantly different. A graphical representation comparing metal concentrations in fish caught in 2003 with others years is shown as box plots in Fig. 4.4.

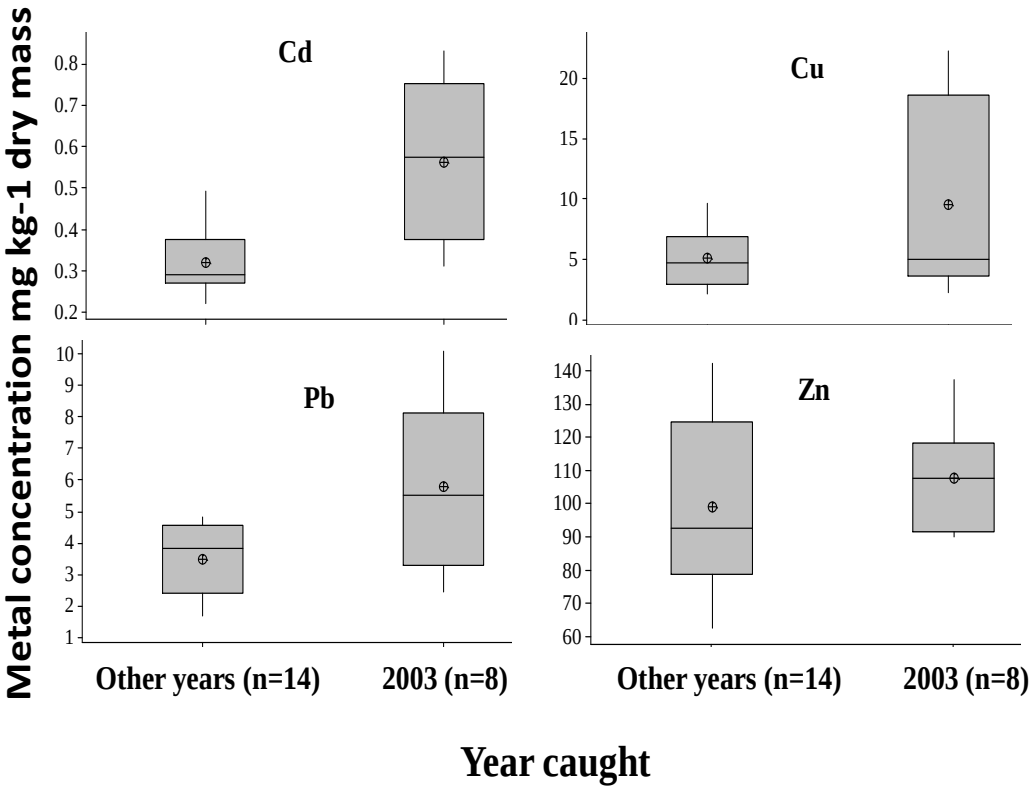


Figure 4.4. Comparison between cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) metal concentrations in sandeels (*Ammodytes marinus*) caught in 2003 and those caught in other years combined, shown as box plots. Each box represents the interquartile range of metal concentrations around the median value, whiskers denote maximum and minimum values. The crossed circle signifies the mean values for each time period when the sandeels were caught.

Although a comparison of the total dry mass of sandeels caught in 2003 with those from other years (Fig. 4.5.) showed the 2003 fish were significantly heavier (Student t-test: t= 2.52, df = 20, p= 0.020). ANOVA did not show a significant difference between years ($F(4,17) = 2.48$, $p = 0.083$). As a result, the higher Cd concentrations in 2003 sandeels compared to those caught in 2004 may be a consequence of the year caught rather than their smaller size.

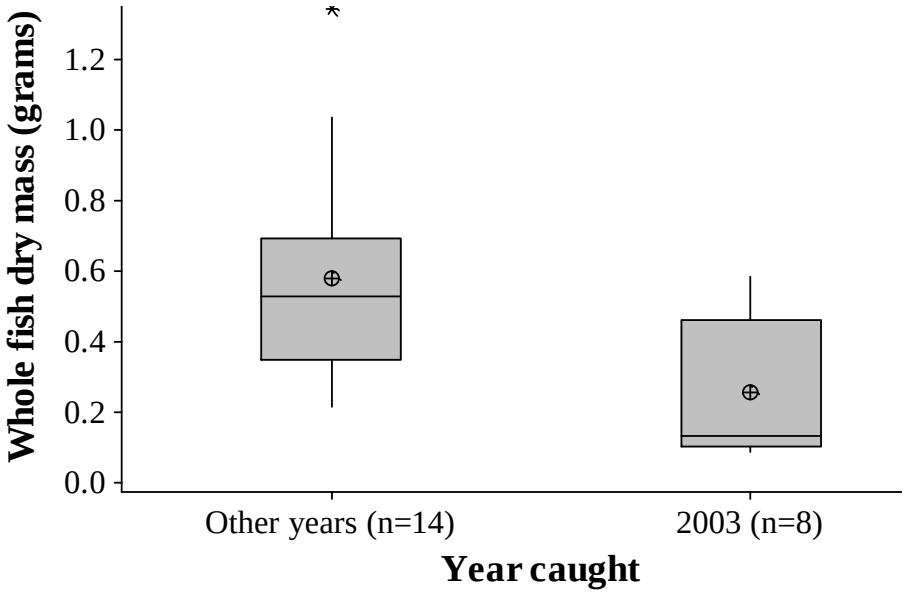


Figure 4.5. Box plot comparing the whole fish dry mass of sandeels (*Ammodytes marinus*) caught in 2003 with those from other years. Each box represents the interquartile range of metal concentrations around the median value, whiskers denote maximum and minimum values and an asterisk any outlier. The crossed circle signifies the mean values for each year caught group.

Table 4.2 compares the median metal concentrations from the earlier 2003 study with samples analysed in this study from the same year and trawl sites, clearly showing higher concentrations of all metals in the earlier sandeel analysis.

Table 4.2. Comparison between median cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) metal concentrations in sandeels (*Ammodytes marinus*) reported by the earlier 2003 study and those from the same year and sites analysed in this study. Metal concentrations in snake pipefish (*Entelurus aequoreus*) from this study and in sprats (*Sprattus sprattus*) from a less polluted fishery study by Amiard et al., (1987), are included for comparison.

Metal	Trawl site	Metal analysis mg kg^{-1} dry mass (median)			
		Earlier study	Current study	Pipefish	Sprats
Cd	Ross Bank	22.4	0.7	< 0.25	0.14
	Inner Farne Bank	12.1	0.4		
Cu	Ross Bank	96.1	4.2	2.0	3.5
	Inner Farne Bank	130.9	13.6		
Pb	Ross Bank	150.8	7.7	< 2.5	0.24
	Inner Farne Bank	137.1	3.6		
Zn	Ross Bank	331.6	110.5	50.0	120
	Inner Farne Bank	452.9	93.4		

4.3.2 Fledgling feather and bone samples

In the feathers (n=6), Cd concentrations were at or below the detection concentration of 0.2 mg kg^{-1}. Cu, Pb, Hg and Zn were all detectable having median (and range) mg kg^{-1} values of 10 (9-12), 4.5 (2-8), 0.46 (0.35-0.52) and 170 (130-240) respectively.
Wing bone samples (n=6) from the same birds only had detectable concentrations of Cu and Zn, the median (and range) values being 2 (1-4) and 220 (160-240) mg kg^{-1} respectively. No significant correlation was shown ($R^2 < 0.1\%$, $p>0.5$) between these metal concentrations in bone and feather samples from each bird.

4.3.3. Fledgling liver samples

Only four of the six liver samples from fledgling terns collected in 2004 were analysed as one was from an adult bird while another had unusually high concentrations of Cu (270 mg kg^{-1}) and Zn (1300 mg kg^{-1}). All livers from both years were below the detection limit for Pb (8mg kg^{-1}), while Cd was not detected in 2004 samples and only detected in 3 of the 2006 samples with a median of 1.1 mg kg^{-1}. Concentrations of Hg in the 2004 samples were significantly lower than 2006 samples ($p<0.05$) with median values of 0.06 and 1.20 mg kg^{-1} respectively. Both Cu and Zn concentrations were not significantly different ($p>0.05$) between the two collection years, with median values for 2004 and 2006 of 28 and 36 mg Cu kg^{-1} and 175 and 200 mg Zn kg^{-1} respectively. A single fledgling puffin liver analysed from 2006 had no detectable Cd or Pb, Hg was at the detection limit (0.06mg kg^{-1}) and Cu and Zn concentrations were 18 and 130 mg kg^{1} respectively.

4.3.4. Whole chick samples

After oven drying it became clear that only the 2004 samples could be classed as whole chicks as despite all the chicks being of a similar age (< 1 week old), the two 2004 chicks had significantly greater dry mass (6.73 and 5.15g) compared to the six 2006 samples (1.99g average mass.), which appeared little more than bones and feathers.
Whole tern chicks (n=2) from 2004 had below detection concentrations of Cd and Pb, with median Cu, Hg and Zn concentrations of 7, 0.22 and 120 mg kg^{-1} respectively. The tern chicks (n=6) collected in 2006 had below detection concentrations of Cd in all but

one sample, with median (and range) Cu, Pb, Hg and Zn concentrations of 16 (10-47), 8 (4-23), 1.15 (0.96-1.6), 155 (140-170) mg kg^{-1} respectively.

4.3.5. Egg shell samples

Egg shells from terns (n=3) and kittiwakes (n=6) had no detectable concentrations of Cd, Pb or Hg. Only one sample from each bird species had detectable concentrations of Zn these being 9 and 14 mg kg^{-1} respectively. Concentrations of Cu in the egg shells between bird species did not appear to differ, both being in the range 2-4 mg kg^{-1}.

4.3.6. AUS samples

The AUS samples extracted from the ethanol-preserved guano of kittiwakes, terns and puffins provided 4, 1 and 5 samples respectively (Table 4.3).

Table 4.3. Cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg) and zinc (Zn) metal concentrations in extracted urate sphere samples (mg kg^{-1} dry mass) from guano of adult seabirds* and pre-hatch seabird chicks† collected on the Farne Islands in 2006.

Bird	Dry mass g.	Cd	Cu	Pb	Hg	Zn
Kittiwake (*Rissa tridactyla*) * (adult and chick)	0.23	1.5	< 0.8	< 8	< 0.06	260
	0.28	2	8	< 4	< 0.06	460
	0.36	0.9	6	< 4	< 0.06	230
	0.43	0.8	7	< 4	< 0.06	180
Tern (*Sterna paradisaea*)*	0.50	< 0.8	14	9	< 0.06	390
Puffin (*Fratercula arctica*)*	0.56	1.5	10	10	0.07	350
	0.33	1.5	10	< 4	< 0.06	310
	0.33	1.5	10	9	< 0.06	340
	0.35	1	11	< 4	< 0.06	300
	0.55	1	7	< 3	< 0.06	310
Kittiwake (*Rissa tridactyla*)†	0.85	<0.2	<2	<2	<.06	7
Tern (*Sterna paradisaea*)†	0.23	<0.2	7	<2	<.06	<4

Only one sample each of kittiwake and tern extracted pre-hatch urine was analysed, being derived from pooled residues from within 10 and 3 egg shells respectively. Because the size of most extracted urine samples was <0.5g, only microscopic examination was used to determine their purity, with all samples appearing to consist entirely of urate spheres. For this reason the urine metal concentrations are reported as mg kg^{-1} dry mass extracted urate spheres rather than mg kg^{-1} uric acid. By assuming these urate sphere samples were approximately 65% uric acid (Casotti and Braun, 2004) a comparison with metal concentrations per mass of uric acid in urate sphere samples from allotment chickens (Chapter 3) is possible (Table 4.4).

Table 4.4. A comparison between copper (Cu), lead (Pb) and zinc (Zn) metal concentrations in extracted urate sphere samples from guano of Farne Island seabirds and allotment chickens. Median values reported as mg kg^{-1} uric acid.

Metal	Farne Island seabird samples*	Chicken samples†		
		Walker Road uncontaminated	Branxton contaminated	Branxton post remediation
Cu	13.8	37.6	65.8	16.0
Pb	0.3	6.6	208.5	147.0
Zn	476.9	250.3	526.0	84.5

*Values derived from assuming urate sphere samples were 65% uric acid

†Data reported in Chapter 3 on metal concentrations in urate sphere samples from domestic chickens.

Cd was detected in all extracted AUS samples except that from tern guano, with concentrations in kittiwake and puffin AUS being similar, with median (and range) concentrations of 1.2 (0.8-2.0) and 1.5 (1.0-1.5) mg kg^{-1} respectively.

Cu was below the detection concentration of 0.8 mg kg^{-1} in one kittiwake urate sphere sample and so estimated as 0.4 mg kg^{-1}. Overall the median (and range) concentration of Cu in kittiwake AUS was 6.5 (0.4-8.0) mg kg^{-1}, this appeared different from puffin AUS which had a median (and range) Cu concentration of 10 (7-11) mg kg^{-1}. The tern sample was higher at 14 mg Cu kg^{-1}.

Pb was neither detected in any kittiwake urate sphere samples nor in 3 out of the 5 puffin samples. The remaining two samples had concentrations of 9 and 10 mg Pb kg^{-1}, and were similar to the tern AUS value of 9 mg Pb kg^{-1}.

Out of all AUS samples only one puffin sample had detectable Hg content, this being just above the limit of detection at 0.07 mg kg^{-1}.

Znwas well represented in extracted AUS from all birds tested, having a value of 390 mg Zn kg^{-1} in the tern urine. Kittiwake, and puffin AUS samples had similar Zn concentrations, with median (and range) values of 245 (180-460) and 310 (300-350) mg Zn kg^{-1} respectively.

In contrast to the AUS samples from the adult birds, only sparing amounts of Cu and Zn were detected in the pre-hatch AUS (Table 4.3).

4.4. Discussion

4.4.1. Fish metal concentrations

Contrary to the reported age accumulation of the non-essential metals (Walsh, 1990), Cd and Pb concentrations declined with body mass increase in sandeels (Fig. 4.1). This could be a result of the reported increase in oil concentrations with size (Harris et al., 2008), masking metal accumulation in their internal organs.

Interpreting the significance of trawl site or year caught on metal concentrations in the sandeels was made difficult because differences in fish size existed between the site and year caught (see Figs. 4.2. and 4.5.). However because the Cu concentrations did not vary significantly with fish size (Fig 4.1.), the lack of variation in Cu between trawl sites or year caught (2003 or other), suggests fish size is the driving factor for any metal concentration variations.

An explanation for this association is that as fish get older and so increase in dry mass, they store more oil (Harris et al., 2008), which should be matched by a reduction in metal concentrations as metals are not stored in fat deposits (Yamazaki et al.,1996). The original data on sandeel metal concentrations was from trawls carried out in 2003 so it was important to determine if the high concentrations were an anomaly of that year alone. The findings from the present study show little difference between years and concentrations measured were far below those reported in the earlier study.

Furthermore, little difference in fish metal concentrations between trawl sites was shown in the present study, although it has been reported that inshore waters are typically more polluted than those further offshore (Walsh, 1990).

As Cu and Zn are essential metals that are under metabolic control, they generally show little tissue variation, even over a wide range of environmental concentrations or different periods of exposure (Walsh, 1990). This is in contrast to the non-essential metals Cd, Pb, Hg and Sn which, being un-regulated, typically show age-related accumulation in tissues (Walsh, 1990; Amiard et al., 1987). Metal concentrations in fish

reported in the literature commonly only refer to muscle values, as these are relevant to human consumption (Burger and Gochfeld, 2005). Whole fish metal values, however, are higher than those found in muscle alone because the liver and other internal organs are sites of metal deposition (Yamazaki et al.,1996; Carpene et al.,1994; Alam et al.,2002). The metal values reported in the present study compare favourably with those reported by Amiard et al., (1987) in whole sprats (*Sprattus sprattus*) from a less polluted region, having mean Cd, Cu, Pb and Zn metal concentrations of 0.14, 3.5, 0.24 and 120 mg kg^{-1} (dry mass) respectively. Furthermore comparable concentrations are reported for fish meal from North Sea whole fish (composed of 38% blue whiting, 30% sandeel, 20% Norway pout and 12% herring), with Cd, Cu, Pb and Zn concentrations of 0.19, 4, 0.09 and 80 mg kg^{-1} (dry mass) respectively (Moren et al.,2006).
In contrast, contrary to the concept of Cu and Zn concentrations being modulated (Walsh, 1990), Unlu and Gumgum, (1993) reported dramatically high mean (n=10) wet mass concentrations of Cu and Zn in samples of liver (829 & 336mg kg^{-1}) and muscle (108 & 59 mg kg^{-1}) respectively, from fish (*Capoeta capoeta umbla*) in a polluted stretch of the Tigris River in Turkey. Although liver concentrations of Cu and Zn in fish (and other biota) are not static, being stored in this organ when in excess (Carpene et al., 1994), the validity of such high concentrations must be called into doubt.
Comparing retested 2003 sandeel samples in the present study with the much higher earlier reported values (Table 4.2.), it may be surmised that sample contamination was responsible, as was experienced to a lesser degree in one batch of fish samples in the present study. The pipefish and sandeel samples in the present study had comparable concentrations of Cu and Zn to those reported in the literature for marine fish. Furthermore the non-essential metals (Cd and Pb) were below detection concentrations in the pipefish and relatively low in the sandeels, which equates with generally less polluted fisheries (Walsh, 1990; Amiard et al., 1987).

4.4.2. Bird urine and diet

As stated earlier, statistical analysis could not be done on the metals in AUS data because sampling reflected technical rather than biological variability. This could have been resolved by directly sampling individual chicks. Furthermore, this would have removed the possible complication of adult and chick diets being different (see below). A further weakness was the relatively small number of samples collected.

In Chapter 3 it was reported that dietary metal variations were reflected in AUS of domestic chickens, relating to the higher Cu concentrations of the commercial ration fed to the Walker Road allotment chickens compared to the predominantly whole grain diet fed to the Branxton chickens (see Table 4.4). As the diet of adult birds and the food they provide for their chicks commonly differs (Barrett et al., 2007), metal exposure and so AUS concentrations may differ between the adult seabirds and their young. The extracted urine samples from terns and puffins were assumed to be from adult birds, so the excreted metals could originate from a different food to that fed to the nestlings. In contrast, the kittiwake AUS samples were a mixture derived from guano of both nestlings and adults. Kittiwakes have a large range (80km) while foraging from the Farne Islands (R.M. Bevan, personal communication). This would allow them to scavenge fishing boat discard and offal (OSPAR, 2000), from boats typically working some distance away, while not necessarily feeding such food to their young (Barrett et al., 2007). This is significant because fish offal has higher metal concentrations than whole fish (Yamazaki et al., 1996) and so ingesting it may result in elevated adult AUS concentrations. The urine metal concentrations reported here may also reflect the persistent excretion of previously accumulated metals in tissues of the adult birds acquired from sites remote from the Farne Islands. Such a persistent excretion was shown in Chapter 3, where six months after the allotment chickens stopped ingesting Pb contaminated soil, they still excreted Pb in their AUS from body deposits (see Table 4.4).

Although the Zn concentrations in the snake pipefish samples and the diet of the control chickens reported in Chapter 3 are similar, with median values of 50 and 57 mg kg^{-1} dry mass respectively, the calculated median AUS Zn concentration in the seabirds of 477mg kg^{-1} uric acid is much higher than the 250 mg kg^{-1} uric acid reported for control chicken AUS (Table 4.4). If it is assumed chickens and seabirds process dietary Zn in a similar manner, this would suggest the adult seabirds are consuming a diet with twice the Zn concentration of the pipefish. This would agree with their eating sandeels, which have a median Zn concentration of 100mg kg^{-1} dry mass. The large quantity of discarded pipefish at the nest sites would confirm the seabirds' preference for sandeels. Although Daunt et al. (2008) reported the sandeel component of nestling seabird diets varied between species, with kittiwakes, puffins and terns, being 87%, 81% and 34% respectively, the nestling terns on the Farne Islands typically have a diet composed of over 90% sandeels (R.M. Bevan , personal communication). This would suggest the

adults and nestlings are mainly feeding on sandeels and the collected urine should reflect the current intake of metals from these fish.

The wide variation in metal concentrations reported in the literature for whole guano from marine birds (Table 4.5.) reflects the diversity of their diet. The high guano metal concentrations reported by Otero Perez (1998) were attributed to the gulls in his study feeding on municipal rubbish tips. Other dietary factors such as eating offal, or older fish and fish at higher trophic levels, would also be expected to increase a bird's metal intake. However it is important to stress again that whole guano levels cannot be taken to represent actual uptake by the bird. This will depend upon the bioavailability of the metal in the diet (Ruby, 2004).

It is widely reported that physiological differences from age, sex and reproductive status can affect the flux of metals within adult birds (Scheuhammer, 1996; Heinz and Hoffman, 2004). As a result many factors in conjunction with their current dietary intake may influence metal concentrations in the seabirds' urine.

Table 4.5. Mean cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg) and zinc (Zn) metal concentrations (mg kg^{-1} dry mass) in seabird whole guano reported in the literature.

Bird	Cd	Cu	Pb	Hg	Zn
Glaucous gull (*Larus hyperboreus*)[a] (n=1)	-	6.25	30.0	-	76
Kittiwake (*Rissa tridactyla*)[a] (n=2)	-	51.2	21.6	-	176
Yellow legged gull *(Larus michahellis*)[b] (n=13)	5.8	60.1	39.9	-	305.1
Red-footed booby *(Sula sula*)[c] (n=12)	6.34	21.1	1.6	107.8	419.4

[a] Headley, (1996); [b] Otero Perez, (1998); [c] Liu et al., (2006)

The prolonged excretion of Pb in AUS after site remediation reported in Chapter 3 (see Table 4.4), would imply that making comparisons between current diet concentrations and whole guano or AUS for non-essential metals (Cd, Pb, Hg and Sn), which accumulate with age (Walsh, 1990), may not be valid. However from the chicken data (Chapter 3), essential metals (Cu and Zn) in AUS appeared to represent current intake values, so differences could reflect dietary intake, although further studies are needed. The metal concentrations in AUS from this study (Table 4.3) cannot be directly compared to the whole guano concentrations (Table 4.5) as was shown in Chapter 3. This is because urine concentrations are entirely composed of excreted metals while the guano is a variable mixture of unabsorbed and excreted metals. Furthermore the

digestive absorption of each metal depends on its bioavailability (Ruby, 2004) also the prevailing body requirements and food concentrations (Mohanna and Nys 1998).
Of relevance to metals passed in the seabird guano is its potential for adding to the soil metal concentrations on the Farne Islands (Headley, 1996; Otero Perez, 1998; Blais et al., 2005; Liu et al., 2006). In many seabird roost sites, where soil is sparse or even absent, such guano deposits may constitute the bulk of the soil, being called ornithogenic soils (Liu et al., 2006). In this study the seabird AUS concentrations of Pb are comparable to those in control chickens fed an uncontaminated diet (Table 4.4), so not significantly contributing to soil concentrations. However the high Zn concentrations (median of 477mg kg^{-1} uric acid) in the seabird AUS may have an impact on local flora as this metal is recognised to be phytotoxic (Gascho and Hubbard, 2006). Furthermore the liberal deposition of guano over the nesting site (R. M. Bevan, personal communication) may contribute, by surface contamination, to the metal concentrations detected in the various samples collected for this study.
The absence of Hg from the adult urine may suggest either the detection concentration was set too high or the majority of Hg in the tissue samples was derived from egg transfer, rather than a current food source (Wenzel et al., 1996). With the exception of the tern sample, Cd is well represented in the seabird AUS and may reflect its association with kidney tissue (Wenzel et al., 1996) and age accumulation in adult birds. Although the tern urine was below the Cd detection concentration of 0.8mg kg^{-1}, this does not preclude it from having significant Cd content. Trace amounts of Cd in some of the tern feather samples may be from surface contamination with guano, while this metal's presence in some fledgling liver samples is probably from the diet. Pipefish had below detection concentrations of Cd ($\leq$0.2mg.kg^{-1}) while sandeels all had detectable concentrations above this although below 1mg kg^{-1}. The Cd in urine from puffins and kittiwakes may suggest excretion of age accumulated deposits in the adult birds or reflect a current intake. In either case, the urine concentrations appear to be a valuable biomarker of exposure to cadmium.
Although the seabird Pb concentrations cannot be fully assessed from liver samples because of the poor detection concentrations (see 4.4.6 below), its lack of detection in the fledgling tern bones, the tissue of predominant deposition (Elliott and Scheuhammer, 1997), suggests a low Pb exposure. This finding is in agreement with the seabird urine having comparable Pb concentrations to unexposed control domestic chickens reported in Chapter 3 (Table 4.4). In this respect because Pb is detectable in

urine samples under apparently low exposure concentrations, urine sampling represents a good method for assessing lead exposure in the seabirds.

Pre-hatch urine

In contrast to adult AUS, Cu and more especially Zn in the extracted pre-hatch AUS are dramatically lower. This may be a conservation strategy as the finite Cu and Zn deposits in the egg are progressively utilised by the developing embryo. This is reflected in the reported fact that liver reserves of both metals become depleted prior to hatching. Also, Cu deposits in the shell are reabsorbed by the avian embryo (Richards, 1997). The reported rapid rise after hatching of liver Cu and Zn concentrations in kittiwake nestlings (Wenzel et al., 1996) may further indicate that chicks hatch with suboptimal concentrations of these essential metals. The possibility of underdeveloped kidneys being responsible for these low metal concentrations is not borne out by research because embryo chicks are reported to have glomerular filtration and tubular reabsorption capabilities equivalent to adult birds from day 5 of incubation (Zemanova et al., 2002). Also, metallothioneins are actively involved in metal transport throughout embryo development (Richards, 1997). The lack of detectable Pb in the pre-hatch AUS would suggest concentrations of this metal detected in the whole chicks were from surface contamination (adult guano). Hg which is excreted in bird guano (Kenow et al., 2007) was not detected in pre-hatch AUS, but this may be a result of up to 93% of egg transferred Hg is sequestered in the down feathers of the chick (Wenzel et al., 1996). How embryo chicks apparently avoid passing metals in their urine, while their kidneys still perform the function of metabolic waste excretion, is an interesting question for future research.

4.4.3. Bird tissue metal concentrations

Although the essential metals Cu and Zn in this study can accumulate in tissues such as liver, they are under homeostatic control and so concentrations have a limited value in determining a bird's exposure (Walsh, 1990). Concentrations of non essential metals (Cd, Pb and Hg) in the bird samples have to be interpreted in terms of both the metal and the tissue being analysed. The reason for this is because metals have tissue specific affinity, for example Hg (Furness et al., 1986) and Sn (Guruge et al., 1996) for feathers and Pb for bones (Elliott and Scheuhammer, 1997). Similarly Hg is passed in eggs (Walsh, 1990) but Cd is not (Burger and Gotchfeld, 1993), and Pb is predominantly a surface contaminant in feathers (Nam et al., 2004). As Cd is not passed in the egg, it

age-accumulates with the growth of the chick at a rate dependant on diet concentrations, Hg in contrast declines over time after hatching if dietary concentrations are low (Wenzel et al.,1996).

Whole chick samples

Meaningful comparison between metal concentrations in the whole chicks from 2004 and 2006 are limited because they were dramatically different in terms of their tissue content (Table 4.5.). As all the chicks were close to newly hatched (≤1 w.o.) they would not have accumulated significant amounts of metals from their diet, compared to the fledgling birds. In this regard the whole chick metal concentrations should approximate to egg content (Wenzel et al., 1996). Also the whole chick samples would principally represent a combination of current diet and age accumulated non-essential metals passed from adult birds into the egg (Wenzel et al., 1996). In contrast liver and bone samples from fledglings, in which the residues from egg transfer are considered negligible (Wenzel et al., 1996), only reflect uptake of metals from the current diet.

The Pb concentrations of the 2006 tern chicks (Table 4.5.) are well above 0.4mg kg^{-1} the value reported in eggs from Pb exposed birds (Trampel et al., 2003) and the median value of 0.57 mg kg^{-1} in eggs from raptors, seabirds and other fish eating birds (Burger, 2002). From Pb having such a low transfer to eggs (Walsh, 1990), the elevated Pb concentrations in the 2006 tern chicks, is most likely to be from surface contamination similar to feathers (Nam et al., 2004). Although UK soils typically have a mean background value of 75 mg Pb kg^{-1} (McGrath and Loveland, 1992), the nearest sediment samples taken from the Ross bank area were determined to have only 16 mg Pb kg^{-1}(Table 4.1). Another source of this apparent Pb contamination could be adult tern guano, which is liberally deposited at the nest sites. Although the tern chicks from both years were a similar age (≤1wo), the 2006 samples were more decomposed and so exposed to a longer period of surface contamination prior to collection. This would explain why in contrast the 2004 tern chicks had undetectable concentrations of Pb (Table 4.5).

The low concentration of Hg in the adult urine samples (<0.06 mg kg^{-1}) would suggest its detection in the whole chicks is not a result of surface guano contamination. Because Hg in freshly hatched chicks is predominantly from the egg content (Wenzel et al.,1996; Becker et al.,1993), whole chicks having a median (and range) value of 1.19 (0.96-1.6) mg kg^{-1} in 2006, and 0.22 (0.20-0.24) mg kg^{-1} in 2004, confirms the presence of Hg in breeding female terns on the Farne Islands. The 2006 concentrations are close to the reported whole egg range of 1.5-6.0 mg Hg kg^{-1} (dry mass equivalent) that reduces egg

viability also embryo and chick survival (Thompson, 1996). However the significantly higher Hg concentrations in 2006 chicks may be explained from having proportionately more feathers, which preferentially sequester Hg (Furness et al., 1986), compared to the less decomposed 2004 chicks.

The lack of detectable Cd in whole chicks (for both years) agrees with this metal's low transfer in eggs, despite Cd being detected in sandeels (see 4.3.1) the likely diet of fledglings leading to detectable concentrations in their livers (see 4.3.3). The low Cd concentrations in whole chicks may also reflect the low concentration in tern urine (<0.8mg Cd kg^{-1}) reducing the potential for surface contamination being a source of Cd in the chick samples.

Fledgling liver samples

Cu and Zn, as essential trace metals under homeostatic control, are reported to stay within a narrow range of concentrations in seabird liver samples (Elliott and Scheuhammer, 1997; Savinov et al., 2003). This was confirmed by concentrations of these metals not being significantly different ($P>0.05$) between years collected or between the single puffin sample and the rest from terns. Zn concentrations in fledgling tern livers from 2006 and 2004 had median (and range) values of 200 (170-230) and 175 (150-200) mg kg^{-1} dry mass respectively. These were similar to concentrations reported in seabirds, typically in the range 100 and 200 mg kg^{-1} dry mass. (Elliott and Scheuhammer,1997; Walsh, 1990). Cu concentrations in the fledgling tern livers were 36 (30-47) and 28 (19-38) mg kg^{-1} dry mass respectively for 2006 and 2004, being similar to reported values of 20 to 30mg kg^{-1} (Elliott and Scheuhammer, 1997; Walsh, 1990). However liver metal concentrations (both essential and non-essential), can be artificially elevated by even short periods of starvation (hours), because of hepatic fat depletion (Evans and Moon, 1981). Periods of starvation are typically associated with inclement weather on the Farne islands (R.M. Bevan personal communication).

Hg concentrations in the fledgling liver samples are well below the maximum normal value of 20 mg kg^{-1} for seabird livers (Walsh, 1990). It however is noteworthy that the Hg concentrations are significantly ($p<0.05$) higher in 2006 liver samples compared to 2004; similarly liver Cd values appear higher, which may indicate increasing dietary exposure to these two metals. Alternatively the body condition of the birds could have varied causing this difference (Evans and Moon, 1981).

Pain et al., (1995) report that avian liver Pb concentrations (dry mass basis) greater than 6mg kg^{-1} are reported to imply some exposure, frank poisoning equating to concentrations above 20mg kg^{-1} and unexposed birds having concentrations around 1

mg kg^{-1}. Consequently, interpreting the results from this study are made difficult with the Pb detection concentration being $\geq$8 mg kg^{-1}, although frank poisoning can be ruled out. This finding is corroborated with the fact that no characteristic behavioural signs of Pb poisoning (Eilser, 2000) have been reported in the seabirds.

Fledgling feather samples

Surface contamination from tern guano could explain the presence of Pb in the 2006 fledgling feathers, despite no detectable Pb in their bones or livers.

From adverse effect concentrations (AEL) reported for Hg, Pb and Cd in feathers of marine birds (Burger and Gochfeld, 2000), the median Hg concentration in fledgling feathers reported here of 0.44 mg kg^{-1}, is well below the AEL of 5 mg kg^{-1}. The median Pb concentration of 5.0mg kg^{-1} is above the AEL of 4 mg kg^{-1}, but may relate to the less severe cleaning method adopted in this study because washing can reduce Pb concentrations in feathers by up to 60% (Scheifler et al., 2006). The Cd concentrations of 0.2 mg kg^{-1} or less in these feathers are well below the AEL of 2 mg kg^{-1}. A weakness of using feathers to monitor metal exposure, even for Hg or Sn concentrations, is that the values only reflects systemic concentrations at the time of feather formation and exposure out with this period will not be shown (Nam et al., 2005). However in this study the feathers are from fledgling birds, in which the feathers were entirely formed while on the Farne islands and so represent a valuable measure of local exposure to Hg and Sn.

Fledgling bone samples

Cu and Zn concentrations were similar to those reported in the literature, with Zn typically 50-100 times that of Cu (Walsh, 1990). Hg and Cd not being associated with bone tissue are predictably below detection concentrations in these samples. Circulating Pb is avidly taken up by avian bone, where it accumulates more than in liver tissue (Elliott and Scheuhammer, 1997), although in acute poisoning, the concentrations may be similar in bone and liver (Paine et al., 2007). Mean Pb concentrations in seabird bone and liver samples were reported as 6.2 and <0.5mg kg^{-1} respectively, without frank poisoning being apparent (Elliott and Scheuhammer, 1997). Bone concentrations of between 20 and 100 mg kg^{-1} are considered to be associated with excessive exposure in a range of bird species (Paine et al., 2007; Ethier et al., 2007). For the measurement of Pb absorption in birds, feathers are of less value than bones (Paine et al.,2007) because of the high degree of surface contamination in feathers (Nam et al., 2004). Consequently the <2 mg Pb kg^{-1} in bones of fledgling terns does not reflect a significant Pb exposure.

Egg shell samples

Egg shell concentrations of Cu and Zn, although near the limit of detection, are similar to values reported for curlews (*Numenius arquata)*, in non-polluted sites (Currie and Valkama, 1998). Egg shell Pb concentrations from eggs laid by birds in Pb contaminated sites were <0.5mg Pb kg^{-1} (Flores and Martins, 1997) and even highly dosed experimental birds only reached egg shell concentrations of 2mg Pb kg^{-1} (Jeng et al., 1997). As a result the detection concentration ($\geq$2mg Pb kg^{-1}) is set too high in this study for egg shells to be of any value in monitoring seabird Pb exposure. Little or no Cd is reportedly deposited in egg shell (Flores and Martins, 1997) similar to its low transfer in the rest of the egg (Scheuhammer, 1987c). Mercury, having little affinity for Ca rich tissues like bone (Nam et al., 2005), is not associated with egg shell, which is in contrast to its high affinity for egg albumin (Heinz and Hoffman, 2004). In conclusion the egg shell metal analysis in this study is of little value for assessing the seabirds' metal exposure.

4.4.4. Analysis for tin in samples

The organic form of tin, tributyl tin oxide (TBTO) has been recorded in a wide range of marine organisms (Kannan and Falandysz, 1997). The source of TBTO in the marine environment is predominantly from anti-fouling paints used on boats (Walker et al., 2001). This pollutant's toxicity is illustrated by its endocrine disruptive effect inducing imposex in the Atlantic dog whelk (*Nucella lapillus)* (Walsh, 1990), with tissue concentrations of 0.2-0.4mg Sn kg^{-1} (dry mass.) as TBTO associated with sterility (Gibbs et al., 1987).

Fish concentrations

Eisler, (2000) reported Sn concentrations in whole marine fish can range from 0.3 to 9.0 mg kg^{-1} (wet mass.). In this study whole pipefish were all below 0.3 mg Sn kg^{-1} (dry mass.) suggesting the lack of butyltin pollution around the Farne Islands.

Bird tissue concentrations

Japanese quail (*Coturnix japonica)* experimentally dosed with TBTO showed reduced enzyme and hormone activity (Coenon et al., 1992), while exposure of egg laying female birds caused embryotoxic effects, reducing hatchability and fertility (Schlatterer et al., 1993). Kannan and Falandysz, (1997) reported elevated butyltin concentrations of 0.35 to 0.87 mg Sn kg^{-1} (wet mass.) in the livers of fish-eating water birds from the

Southern Baltic Sea resulting from TBTO pollution. Livers from cormorants (*Phalacrocorax carbo)* living on Biwa Lake in Japan (noted for its TBTO pollution), were reported to have elevated butyltin concentrations ranging from 0.14 to 1.01 mg Sn kg^{-1} (wet mass.) (Guruge et al., 1996). As all the fledgling tern liver samples in the present study had concentrations below 0.075 mg Sn kg^{-1} (wet mass.) calculated by assuming 80% moisture in the livers (Kannan et al., 1998), this would suggest TBTO is not a significant pollutant in the Farne Island sea area.

Butyltin exposed cormorants from Lake Biwa, Japan showed elevated feather concentrations (median 0.30 range 0.15-0.82 mg Sn kg^{-1} wet mass.), which correlated with high concentrations in the whole body, suggesting feathers can be used as a non-destructive biomonitor of avian butyltin exposure (Guruge et al., 1996). Cormorants accumulated 20-30% of their butyltin body burden in feathers, suggesting the moult aids detoxification in these birds (Guruge et al., 1996). The preferential deposition of butyltin in feathers was also reported by Senthilkumar et al., (1999b), recording butyltin concentrations in feathers from birds in Southern India of <0.3mg Sn kg^{-1} (wet mass.). They commented that these birds were less contaminated than birds from other parts of the world. The finding of the present study that tern feathers had less than 0.3mg Sn kg^{-1} (dry mass) would also suggest butyltin is not a significant pollutant in the Farne Island sea area.

Urine concentrations

The analysis of urine is used to monitor organotin exposure in humans (CDC, 2005). For this reason AUS may be of value to determine avian exposure levels; the results of the present study may indicate that low exposure equates to AUS concentrations below 0.3mg Sn kg^{-1} dry mass.

4.5 Conclusions

This study clearly showed the earlier reported metal concentrations in sandeels caught in 2003 from around the Farne Islands (Table 4.1.) were erroneously high. Compared to the earlier data, the fish results from the present study better resemble values reported for fish metal concentrations in the literature (Furness, 1993; Amiard et al., 1987). In the light of this, the seabirds of the Farne Islands are not being exposed to elevated concentrations of metals from the sandeels in their diet.

A factor which should have caused suspicion over the accuracy of the earlier results was the high values for the essential metals Cu and Zn, which are usually metabolically

controlled to within narrow physiological concentrations across diverse species (Walsh, 1990).

The low metal exposure to the seabirds was also confirmed by concentrations in the various tissues analysed in this study being similar to unexposed birds reported in the literature.

Without having control seabirds to compare AUS metal values with, validation of using the AUS as a sampling method to measure the seabirds' exposure to metals is not possible. However if guano samples had been collected from individual nestlings along with crop sampling (Sutherland et al., 2004) this could have been a useful study into using AUS to monitor metal intake. From the point of view of the potential use of AUS for biomonitoring metal pollution, it is encouraging that metals could be detected in AUS (despite high minimum detection concentrations), even when no pollution is suspectedbecause higher AUS metal concentrations would be expected under conditions of frank exposure.

Chapter 5.

Measuring corticosterone in avian urine

5.1. Introduction

5.1.1. Measuring hormones in bird guano

Over the past two decades the non-invasive measurement of excreted hormone metabolites in animal faeces, including bird guano, has gained popularity (Palme, 2005). Guano steroid monitoring offers a powerful alternative to blood sampling and the associated handling which can trigger a stress reaction detrimental to the bird. The potential value of this technique for avian physiology studies is shown by its capacity to determine a bird's endocrine status. The guano concentrations of excreted sex hormones such as oestrogen, progesterone and testosterone, have been correlated with avian breeding cycles (Bishop and Hall, 1991; Kofuji et al., 1993; Tell, 1997; Sorato and Kotrschal, 2006). Furthermore, guano concentrations of corticosterone (the avian stress hormone), have been shown to reflect changes in the hypothalamus pituitary adrenal (HPA) axis in birds (Goymann et al., 2002; Baltic et al., 2005). However, interpreting the results of such bird studies can be problematic because of the varied composition of guano, which is a mixture of faeces and urine (Klasing, 2005). It is reported that ambient temperature, food composition and other factors that lead to differences in faeces production may affect the concentration of excreted hormones in guano (Goymann et al., 2006). As a result, although guano hormone concentrations are commonly expressed by dry mass (Wasser et al., 2000), it is now thought the total quantity of excreted hormone over time in guano is a more accurate measure of a bird's hormone status (Goymann et al., 2006; Carlsson et al., 2009). This finding seriously restricts guano hormone studies in free ranging birds, because it is impractical to collect their total guano output over a defined time period (Goymann et al., 2006).

A further complication of measuring the faecal excreted hormones in guano is that they constitute a mixture of several metabolites, with little or no parent hormone (Hirschenhauser et al., 2005). These metabolites are shown to differ between male and female birds of the same species (Rettenbacher et al., 2004) also with diet (Goymann et al., 2006). As a consequence, measuring the complex mixture of hormone metabolites

in guano and relating these values to circulating hormone concentrations is problematic (Goymann, 2005).

To overcome the above issues, measuring excreted hormones in the urine fraction of bird guano may avoid the disadvantages inherent in using the faecal component. Such a method would be comparable to the analysis of mammalian urine samples, which are successfully used for assessing an animal's endocrine activity (Touma and Palme, 2005).

5.1.2. Avian urine as a source for excreted hormones

Avian urine has been shown to contain excreted hormones (Hiebert et al., 2000; Rettenbacher et al., 2004; Wasser and Hunt, 2005). Avian urine is composed of a suspension of urate spheres, which contain concentric layers of serum albumin derived from the bird's bloodstream (Janes and Braun, 1997). This major blood protein transports many substances including hormones (Peters, 1996). As a result the albumin would be expected to transfer bound hormones from the blood into the avian urate spheres (AUS). The liquid fraction of avian urine, described as cloacal fluid (CF), has been used to identify excreted corticosterone in hummingbirds (Hiebert et al., 2000). This technique presents several disadvantages being restricted to birds like hummingbirds on predominantly fluid diets which pass more liquid urine compared to other species. The liquid CF can also soak away and dry, making collection problematic. In contrast, the solid fraction of urine composed of AUS is more stable and suitable for collection in the wild. Furthermore, CF has the disadvantage of being contaminated by faecal material as was shown by Hiebert et al., (2000) when oral steroid was given. Similarly, faecal contamination often causes blood, glucose and protein to be detected in CF samples used in clinical diagnostics (Tschopp et al., 2007).

In contrast to faecal hormones, which are predominantly conjugated metabolites (Hirschenhauser et al., 2005), AUS hormones are likely to be in the parent form, making specific analysis easier. Furthermore, unlike faecal excreted hormones, which are highly modified by digestive processes (Klasing, 2005), urine excreted hormones may be protected within the AUS and remain unchanged between their renal formation and being passed in guano (Janes and Braun, 1997). A possible reason why urinary excreted parent hormones have not been detected in whole guano is because the

hormone metabolites are extracted using alcohol (Palme, 2005), in which the AUS will remain intact (Drees and Manu, 1996).

In the literature two radio-labelling infusion studies show avian urine contains excreted steroids (Wasser and Hunt, 2005; Rettenbacher et al., 2004). However Wasser and Hunt (2005) reported the solid urates were devoid of steroid metabolites while Rettenbacher et al., (2004) detected no parent steroid molecules. These finding contradict the general hypothesis of this thesis which predicts parent hormone should be detected in extracts of the AUS.

Wasser and Hunt (2005) reported that the solid urates after separation from the liquid part of the urine in two owl species had negligible radio-labelled steroid metabolites following infusion experiments. However these solid urates were not examined for morphology and could have been composed of hydrolysed AUS following contact with the water that Wasser and Hunt (2005) used to separate the urine from the faeces. The addition of water to AUS is reported to cause spontaneous re-crystallization with the subsequent release of incorporated solutes (Drees and Manu, 1996). Consequently the radio-labelled steroid metabolites in the AUS could have been released into the separately-analysed liquid phase of the urine where they were detected in significant amounts (Wasser and Hunt, 2005). Furthermore Wasser and Hunt (2005) did not determine the metabolite profile in the liquid urine fraction only the faecal fraction, where similar to other authors (Hirschenhauser et al., 2005), they detected no parent steroid molecules.

In a similar infusion experiment in domestic chickens (Rettenbacher et al., 2004), using radio-labelled corticosterone, the urine fraction (solid and liquid combined) was also shown to have substantial quantities of radio-labelled metabolites, although it was not analysed for which specific metabolites. This was carried out on whole-guano samples using an alcohol extraction method (methanol 60% v/v) that preserves the AUS structure (Drees and Manu, 1996) and so prevented steroid analysis of the AUS. Furthermore the total recovery rate of radioactivity in this study ranged from 52 to 97% which also supports the hypothesis that some (possibly parent hormone) was undetected because intact AUS were discarded prior to the analysis.

5.1.3. Validation of hormone measurements on AUS extracts

As blood hormone concentrations are considered a true measure of hormone status in an animal (Touma and Palme, 2005) it was necessary to correlate AUS concentrations with circulating blood concentrations of corticosterone. However the interpretation of blood hormone concentrations is complicated in two ways:

Firstly, hormones in blood are composed of bound and unbound fractions, with the latter, rather than total amount, being thought responsible for hormone activity (Romero et al., 2006). Consequently hormone analysis of blood samples should include a measure of hormone binding proteins in addition to total hormone concentrations (Breuner et al., 2006).

Secondly, some hormones are released in a pulsatile manner (ultradian rhythms); this is shown as repeating peaks and troughs in sequentially taken blood samples (Young et al., 2004). As a result the hormone concentration in a single blood sample will depend upon the point at which the pulse is sampled.

These complications make correlating excreta hormone concentrations with blood concentrations problematic. As a result, *biological* or *physiological* techniques, as defined by Goymann (2005), are preferable when validating the measurement of hormones in excreta (Goymann, 2005). '*Biological*' validation depends upon measured hormone changes reflecting normal biological processes, such as the circadian or daily corticosterone changes (Breuner et al., 1999). '*Physiological*' validation is based upon showing hormone changes in response to pharmacological agents, such as the suppression of corticosterone concentrations by dexamethasone administration (Westerhof, 1998).

To validate measuring corticosterone concentrations in AUS, one biological and three physiological techniques were explored in this study. The *biological* method was to show the diurnal rhythm of corticosterone concentrations (Breuner et al., 1999). The *physiological* methods involved altering circulating corticosterone concentrations by (i) ACTH stimulation (Goymann et al., 2002), (ii) dexamethasone suppression (Westerhof, 1998) and (iii) the oral administration of exogenous corticosterone (Breuner et al., 1998).

To facilitate this, a series of experiments on wild-caught great tits (*Parus major*) maintained in laboratory conditions was devised. In these experiments, blood and AUS

corticosterone concentrations were measured concurrently in individual great tits. Furthermore, to compare this proposed AUS analysis technique with a currently used whole guano protocol, guano samples were concurrently analysed for corticosterone using the method reported by Goymann et al., (2002).

5.1.4. Summary and specific aims

In summary the non-invasive measurement of bird hormones is a powerful tool for avian physiological studies. However the current techniques which measure excreted hormones in whole guano have serious drawbacks. It is hypothesised that measurement of hormone concentrations in AUS may be a viable alternative but first a suitable extraction and hormone analysis method must be developed to enable subsequent validation.

Consequently the specific aims of this chapter were to:

1. Develop a method to extract and analyse corticosterone from AUS.
2. Validate the proposed avian urine analysis method.

5.2. Materials and methods

5.2.1. Great tit subjects and housing

Eight great tits (four male and four females), were captured from the wild on 19th March 2007, under Home Office Project License number 60/3608. They were kept in a windowless room, approximately 3 metres square, on a fixed photoperiod of 06:15 to 20:15 BST. Ambient temperature was thermostatically controlled at 18 ± 2^{0}C. The great tits were housed individually in wire mesh cages of height 45cm and floor area 45cm x

72cm set on wall mounted shelves around the room. The diet of the birds was kept the same throughout their captivity (Table 5.1.).

Table 5.1. Ration fed to the captive great tits (*Parus major*) in this study.

Bogena® Universal Food	1 x 5mL scoop
Peanuts	5
Mealworms	6
Wax moth larvae	2
Sunflower seeds	Pinch
Apple or pear	$1/8^{th}$ cut segment
Water with added Vit A*	200mL + 2 drops vitamin supplement
** Blue tits, in particular are susceptible to vitamin A deficiency (Hawkins et al., 2001).*	

Each cage had two perches running front to back. Awater dispenser and a food bowl were attached to the front wall of the cage. When guano samples were not being collected, pieces of apple, some foliage and a water bath were provided to enrich the bird's environment.

5.2.2. Guano sampling techniques

Because initial capture can alter stress physiology (Dickens et al., 2009) no guano collection was carried out on the great tits in the two months after capture. This allowed the birds to acclimatize to the daily cleaning and feeding routine which took place at 09:00 hrs each morning.

Cellophane sheets were placed in the bottom of each cage to collect guano from each bird at timed intervals of 2 hours. It was found 2 hours provided adequate guano to extract enough urine (approximately 50mg dry mass.) for hormone analysis. However this time period was increased when guano production decreased such as during weighing, blood sampling or drug administration. If the whole guano was to be stored it was folded up in the cellophane collection sheet and frozen (-80^0C). For immediate AUS hormone extraction the guano was scraped from each sheet, using a disposable plastic knife, into individual glass sample pots containing 5mL of GPR absolute ethanol. Any AUS adhering to the sheet were suspended in a few drops of ethanol and transferred using a glass pipette.

5.2.3. Blood sampling procedure

Blood sampling was performed under a Home Office Personal Licence (PIL 60/11083). Methods for blood sampling birds are described in the literature (Phillips, 1999; Hawkins et al., 2001). Although these authors suggest the right jugular vein is a suitable site in smaller birds such as great tits, it was found the cutaneous ulnar (wing) vein was preferable. This was concluded after one female great tit suffered a fatal haemorrhage during jugular blood sampling, resulting in seven birds remaining for this study.

Furthermore, sterile water was used to part the feathers rather than alcohol (Hawkins et al., 2001) because the resulting vasoconstriction made the wing vein impossible to see. It is recommended by Hawkins et al., (2001) that for a one-off blood sample 0.5mL of blood can be safely taken for every 100g body mass of bird. While Phillips, (1999) reports twice this amount (1% of body mass) can safely be withdrawn without ill effects. As these birds were between 15 and 20 g, to err on the side of caution the blood volume collected was restricted to 50µL taken once in any two week period. This was also within the acceptable limit for sample volume defined in the Project Licence as 1% of body mass in any 28 day period. The birds were caught by turning the aviary lights off, and using minimal light such as a chink in the door or a small torch. The bird was immediately taken to a separate room out of sight or hearing of the others, for bleeding.

Blood was taken by puncturing the wing vein with a fine hypodermic needle (26-gauge) with the drop that welled up being collected in a heparinised micro-haematocrit tube (Fisher Scientific, UK). The time between entering the aviary to catch the bird and blood collection was kept to less than 3 min, to ensure that plasma corticosterone concentrations were not elevated due to capture stress (Wingfield et al., 1982). The tube was plugged with Cristaseal® and kept upright on ice until centrifuged at 15,000g for 15 min. The tube was then snapped to separate the plasma that was then expelled into a 0.5mL Eppendorf tube and stored frozen (-20^0C) for later analysis.

Haemostasis was achieved immediately after blood sampling by holding cotton wool to the puncture site for 1 minute. After this, if no further bleeding was visible, the bird was returned to its cage.

5.2.4. AUS sample preparation and hormone extraction

AUS extraction from whole guano

The guano samples collected in GPR absolute ethanol were homogenised using a glass rod, vortexed briefly and the suspended AUS pipetted off into 2mL Eppendorf tubes leaving the faecal sediment behind. These Eppendorf tubes were then centrifuged at 2,500 x g for two min, after which the ethanol supernatant was discarded. The residue was then washed twice using fresh ethanol by briefly vortexing then centrifuging (2,500 x g for 2min) and discarding the supernatant each time. The final residue was air dried to constant mass and represented the AUS sample from a single bird over one collection period.

Guano samples that had been stored frozen were defrosted at room temperature on the cellophane collection sheets. If only the AUS fraction was to be analysed the same procedure of washing with GPR ethanol as described above was followed. If however the whole guano sample was to be analysed for both faecal and AUS corticosterone, the guano was scraped (using a disposable plastic knife) onto an aluminium foil sheet (10cm x 10cm) on top of a heat block set at low heat (80^0C) and thoroughly mixed while being dried to constant mass. The dry mass of each whole guano sample was recorded (see Fig.5.6). This dried guano sample was suitable for faecal corticosterone analysis using the method of Goymann et al., (2002). As the residue from this faecal analysis contained intact AUS, it was used to measure the corticosterone concentration in the AUS from the same guano sample.

Hormone extraction from AUS samples

Between 30-50mg of air dried AUS was accurately weighed in a 2mL Eppendorf tube using a Sartorius LE225D (Epsom, UK) balance, to this was added 2mL 0.5M HCl, mixed thoroughly by vortexing (10 sec) and then placed in a sonication bath (Ultrasonics Ltd, Hove, UK) for 10mins. The hydrolysed contents were then transferred to a ground glass stoppered test tube and shaken with 5mL ether for 15 min. The mixture was centrifuged for 2 min at 2000x g in a refrigerated centrifuge (4^0C) to aid phase

separation, then snap frozen in a dry ice/ethanol bath. To neutralise residual acid in the ether layer it was decanted into a fresh tube containing an equal volume of 1% Na_2CO_3 vortexed to mix thoroughly, then rested or centrifuged to allow phase separation prior to again snap freezing (dry ice/ethanol bath). The ether layer was decanted into an open glass test tube and the solvent fully evaporated in a fume cupboard using an air manifold. The residue was re-suspended in 200µL of buffer and 50µL of 'Caldil' from the corticosterone ELISA kit. This solvent represented the zero standard of the ELISA kit and the small final volumes constituted a concentration step in the extraction. Re-suspension was aided by placing the glass test tubes in a sonication bath for 5mins. The contents were then transferred to 0.5mL Eppendorf tubes and fridge stored (0-4^{0}C) prior to hormone analysis for corticosterone, using the ELISA test kit.

Uric acid analysis of AUS samples

The uric acid content of individual AUS samples was determined using a combination of an extraction protocol adapted from Adeola and Rogler, (1994) and the spectrophotometric method of Van Handel (1975). In brief, the remaining aqueous and solid residue from ether extraction was allowed to thaw, then neutralised by adding solid sodium bicarbonate until effervescence (from liberated CO_2) stopped. To this 10mL of 0.5% Li_2CO_3 was added, vortexed to mix thoroughly and then incubated in a boiling water-bath for 10 min. After allowing it to cool, the entire solution was transferred to a 100mL volumetric flask and made up to volume with 18MΩ water. After thorough mixing approximately 5mL was syringe-filtered (Whatman Puradisc, UK) and analysed for uric acid using the spectrophotometric method of Van Handel (1975). The uric acid content of each urine sample (dry mass) was determined using a uric acid standard calibration curve and calculation of the dilution factors.

5.2.5. Hormone analysis of samples

ELISA method

Whole guano, AUS extracts and plasma samples were analysed for corticosterone content using the OCTEA HS ELISA kit (IDS ltd, Boldon, UK). The kit was used in

accordance with the manufacturer's protocol. The ELISA multi-well plates were read using a Spectra Max-Plus 384 micro-plate reader with Soft Max-Pro software (Molecular Devices, Sunnyvale, CA, USA). Absorbance data was analysed using the programme Fig-P for Windows version 2.7 (Biosoft Ltd. Cambridge, UK). For each analysis a calibration curve was constructed from the kit standards and two control samples (high and low) were used to determine intra- and inter-assay accuracy. Specificity of the analysis for corticosterone in the urate sphere extracts was tested by showing parallelism between the displacement curves of serial diluted extracts and the corticosterone standard (Goymann et al., 2002). The ELISA kit detection limits were calculated for each analysis from the concentration corresponding to the mean absorbance of the zero standards minus two standard deviations. Samples were assayed in duplicate and concentrations were expressed as nanograms per gram uric acid. The ELISA antibody cross reactivity (at 50% binding of zero calibrator) provided by the manufacturer stated the following cross-reactivity values: 11-Desoxycorticosterone 18.5%, 11-Dehydrocorticosterone 2.0%, Aldosterone 0.26%, Dexamethasone 0.11%, Cortisol and Progesterone 0.09%, with other analytes at or below 0.01%. Values were not given for the common glucuronate or sulphate corticosterone metabolites and so were assumed to be very cross reactive. The significance of the low cross-reactivity to dexamethasone is important in regard to its use in the suppression experiment (see 5.3.6.).

Other analytical methods used for corticosterone analysis

To compare analytical techniques, ether extracts derived from several great tit urine samples were reconstituted in mobile phase for RP-HPLC detection using the method adapted from Wong et al., (1994). Initial analysis showed such samples were highly contaminated, making detection impractical. In an attempt to resolve this problem the ether extracts were cleaned up using a method adapted from Hunt et al. (2006) prior to RP-HPLC analysis.

A further technique to clean up the urine samples was to collect standard derived, timed samples eluting from the RP-HPLC column ('heart cut'). These samples were analysed by ELISA also LC-MS/MS using a method similar to Samtani and Jusko, (2007) for parent corticosterone detection.

5.2.6. Experimental procedures on great tits.

Sex difference in AUS corticosterone concentrations

At the outset of this study, four birds of each sex were selected. However this design became unbalanced following one female dying. In the light of sex influencing the profile of excreted corticosterone metabolites (Rettenbacher et al., 2004; Goymann et al., 2002) it was necessary to determine if sex had a significant effect on AUS corticosterone concentrations and so determine if sex needed to be considered in any comparative treatment study.

Diurnal changes of corticosterone concentrations

In this experiment guano samples were collected at 3-hour intervals because overnight guano production was expected to decline from the birds not feeding in the dark. The first sample was collected between 15:00hr and 18:00hr on day 1 and the last between 12:00hr and 15:00hr on day 2., These were immediately frozen (-80^{0}C) for later analysis. The collection of guano in the dark period at 21:00hr, 24:00hr and 03:00hr was facilitated by a low intensity head torch with a cyan coloured filter (Romero and Rich, 2007), during which the birds remained immobile and silent. All the guano samples collected in this experiment were later defrosted, dried and weighed, which provided a measure of guano production by each bird over the 24hr period. Only the first guano samples after lights-on (06:00-09:00hrs) and the late afternoon samples (15:00-18:00hrs) were analysed for corticosterone because they were expected to contain the extreme high and low concentrations respectively of basal corticosterone over the diurnal cycle (Breuner et al., 1999). These samples were analysed concurrently for both faecal and urine excreted corticosterone, using the method for whole guano reported by Goymann et al., (2002) and the one described here for AUS respectively.

ACTH stimulation of corticosterone concentrations

The administration of exogenous adrenocorticotropic hormone (ACTH) has been widely used in birds to stimulate the increased release of corticosterone from the adrenal cortex and so elevate circulating blood concentrations (Astheimer et al., 1994; Wada et al., 2007). The ACTH analogues used in this study were 1-24 segment ACTH (Sigma) or

Synacthen (Alliance). For both, a 1µg dose was given via intramuscular (IM) injection in the pectoral muscle. From the reported use of ACTH in birds (Spelman et al.,1995; Goymann et al.,2002; Rettenbacher et al.,2004; Mostle et al.,2005) a blood peak of corticosterone 30-60 min following ACTH injection was expected, with a urine peak followed by a faecal peak over the next hour. Control birds not given ACTH received a similar volume of sterile normal saline IM.

Guano was collected prior to ACTH or saline administration at 11:00h and blood samples were taken at 2 or 4 hours following the injections. To determine if blood sampling following ACTH injection would prolong the release of corticosterone (Noirault et al., 1999), some birds were not bled following ACTH administration. Guano was collected up to 17:00hr (see Table 5.2. for scheme).

Table 5.2. Scheme for ACTH stimulation and sample collection in great tits (*Parus major*).

Time	Week 1	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6	Bird 7
09:00	Load cellophane							
11:00	Remove sheet 1	Inj ACTH	Inj ACTH	Inj ACTH	Inj saline	Inj saline	Inj ACTH	Inj ACTH
13:00	Remove sheet 2	Bleed						
15:00	Remove sheet 3		Bleed	Bleed	Bleed	Bleed		
17:00	Remove sheet 4							

Time	Week 2	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5	Bird 6	Bird 7
09:00	Load cellophane							
11:00	Remove sheet 1	Inj ACTH	Inj saline	Inj saline	Inj ACTH	Inj ACTH	Inj ACTH	Inj ACTH
13:00	Remove sheet 2		Bleed	Bleed			Bleed	Bleed
15:00	Remove sheet 3	Bleed						
17:00	Remove sheet 4							

Because the initial results from this experiment were inconclusive from possibly endogenous stress responses causing 'noise' in the measured hormone concentrations (Wilson and Holberton, 2001), the method was modified. This involved the oral administration of dexamethasone (see below) prior to ACTH stimulation, to prevent such endogenous stress responses masking the effect of ACTH on AUS corticosterone concentrations.

Dexamethasone suppression of corticosterone levels

In an adaption of the method described by Wilson and Holberton (2001), a 5mg/kg BW oral dose of dexamethasone was thought suitable and safe to be given to the great tits.

This was administered as a single oral dose of 100µg per 20g bird, using 20µL of 5mg/mL dexamethasone in DMSO inoculated into a wax moth (*Galleria mellonella*) larva fed to each bird. The dexamethasone was administered at least two hours prior to suppression being required (Vanmontford et al., 1997) or even the day before as the effect is reported to persist for several days (Westerhof, 1998). To avoid prolonged or additive suppression, which can be fatal in birds (Astheimer et al., 1994), dexamethasone was not given more than once a week to the great tits.

Oral administration of corticosterone

In this protocol, an oral dose of 20µg corticosterone was given to four of the great tits, by feeding a mealworm (*Tenebrio molitor)* injected with 20µL of 1mg/mL corticosterone in DMSO as previously described (Breuner, et al., 1998; Saldanha et al., 2000). The remaining three birds were given mealworms injected with 20µL DMSO as a control treatment. From the study by Breuner, et al., (1998) it was expected that the birds would show a rapid increase in blood corticosterone (within 7 min), returning to a resting concentration after one hour. Consequently the time interval between feeding the spiked food and taking a blood sample was critical to detecting the blood corticosterone peak and was kept between 20 and 30 min. Guano samples were collected at two-hourly intervals from each bird comprising two collections before, and three after, the time of treatment.

5.2.7. Data analysis

Minitab (version 15) and Sigma plot (version 11) programmes were used for statistical analyses and graphical output. Parametric analysis using the paired T test was carried out on data from individual birds and the two sample T test on birds as a group, after confirming the data were normally distributed (Kolmogorov-Smirnov Test) and had equal variance (Levene's Test). For data not normally distributed, the Mann-Whitney U test was used to determine significant differences between data sets. ANOVA was performed on the 24hr guano data to compare urine and whole guano production for each 3hr sampling period. P values less than 0.05 were taken to show a statistically significant difference.

5.3. Results

5.3.1. Specificity of the ELISA analysis for corticosterone

The test for parallelism, used to show specificity of the ELISA test for corticosterone (Goymann, 2005), between serial dilutions of AUS extracts and a corticosterone standard showed a close approximation to each other. However whole guano extracts produced using the faecal extraction method by Goymann et al., (2002) did not show parallelism (Fig.5.1.).

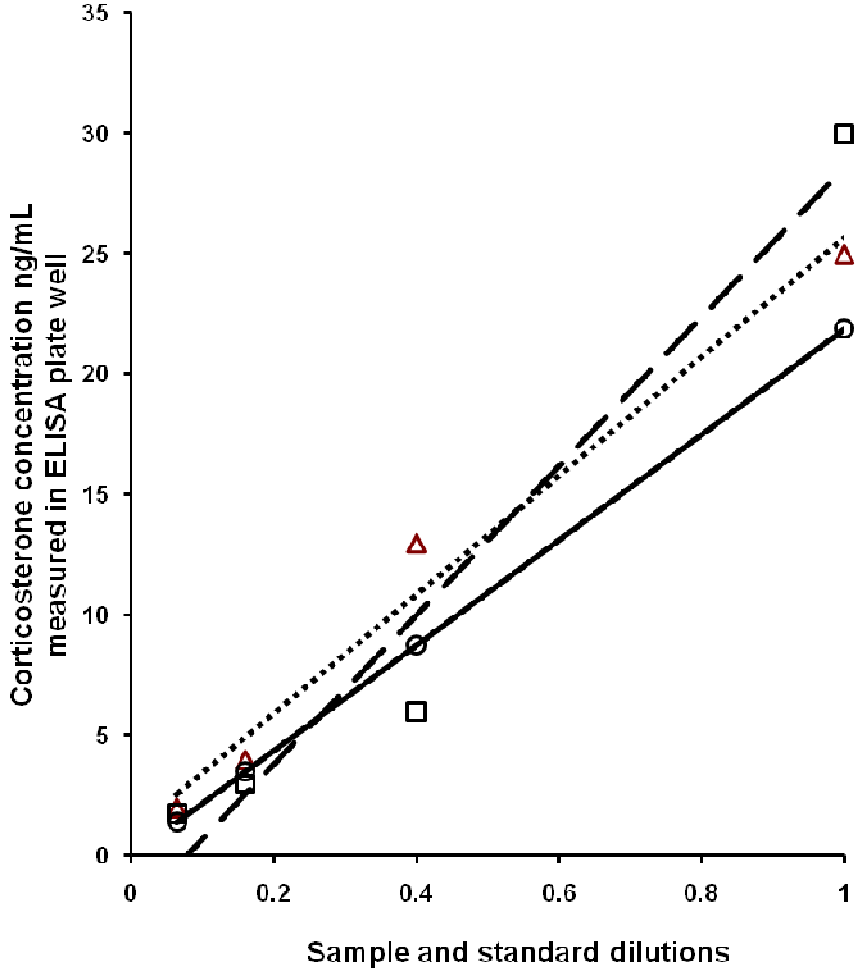


Figure 5.1. Graph showing test for parallelism of trend lines between serial dilutions of a corticosterone standard (circle, solid line) and extracts from either urate spheres (triangle, dotted line), using the reported method here or whole guano (square, dashed line), using the method by Goymann et al., (2002).

5.3.2. Sex difference between corticosterone concentrations in AUS samples

The corticosterone concentrations in male and female AUS samples (15 of each) were collected over a single day and compared (Fig.5.2.). On this occasion the AUS from male great tits had significantly higher corticosterone concentrations than AUS from the female birds ($t = 2.40$, $df = 28$, $p = 0.023$). However in a later analysis with a smaller sample size (8 male and 6 female samples), both faecal and AUS corticosterone concentrations failed to show this relationship (Fig.5.3.). The Mann-Whitney U test showed no significant difference in steroid concentrations in faeces ($p = 0.282$) or AUS ($p = 0.852$) between sexes.

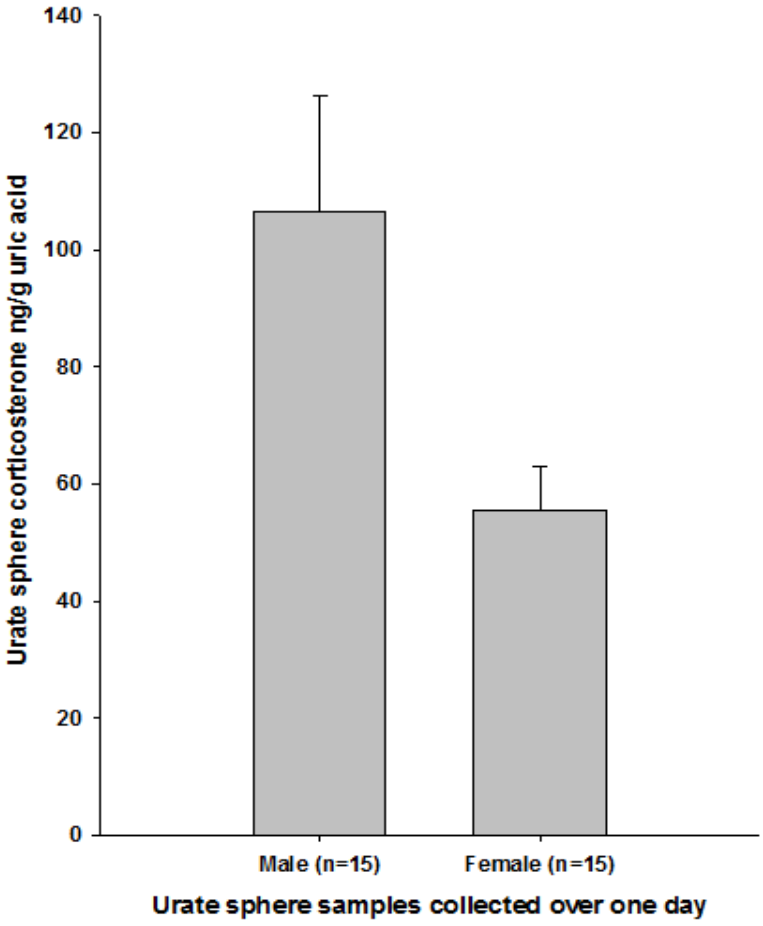


Figure 5.2. Corticosterone concentrations (ng/g uric acid), measured by ELISA, in 15 extracted urate sphere samples from four male and four female great tits (*Parus major*) collected over a single day. Mean values with standard error bar.

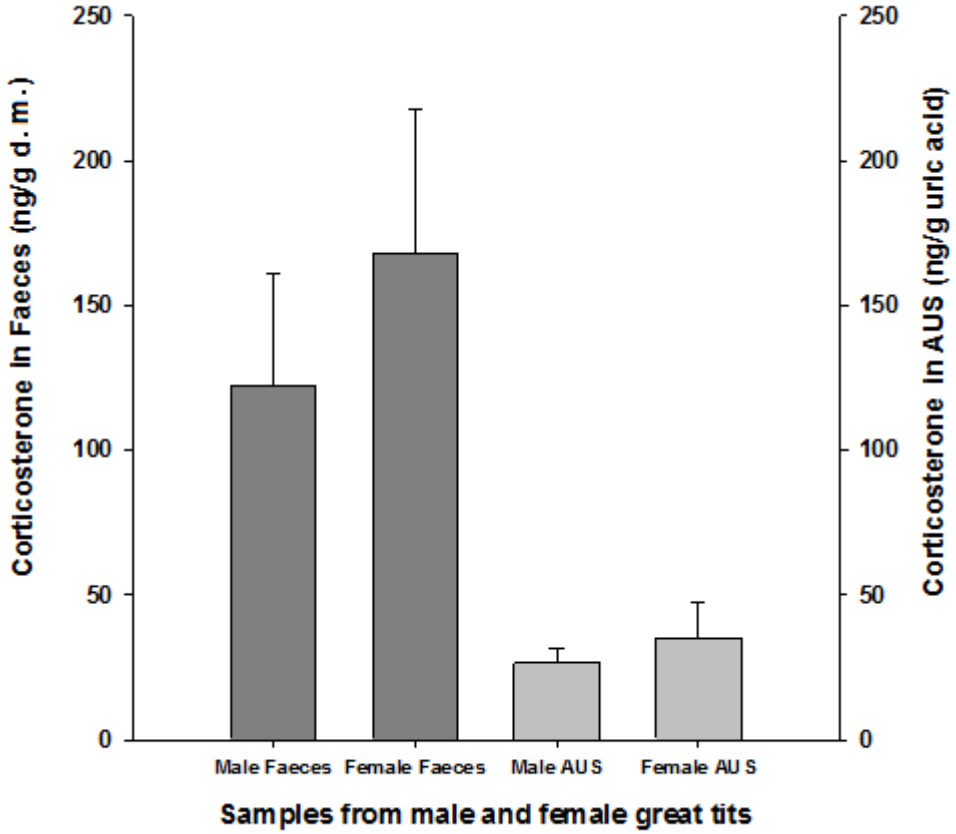


Figure 5.3. Corticosterone concentrations measured by ELISA, in faeces (dark grey) using the method by Goymann et al., (2002) and in avian urate spheres (light grey), from male (8 samples) and female (6 samples) great tits (*Parus major*). Mean values with standard error bar.

5.3.3. Diurnal changes in excreted corticosterone concentrations

Corticosterone concentrations were measured concurrently in faeces and AUS from the great tit guano samples collected in the morning and late afternoon (Fig.5.4.). There was a significantly higher corticosterone concentration in the morning faecal samples than the late afternoon samples ($t= 2.82$, $df = 12$, $p = 0.015$). Although a similar trend was apparent in the urate sphere samples it was not statistically significant ($t= 2.01$, $df = 12$, $p = 0.068$).

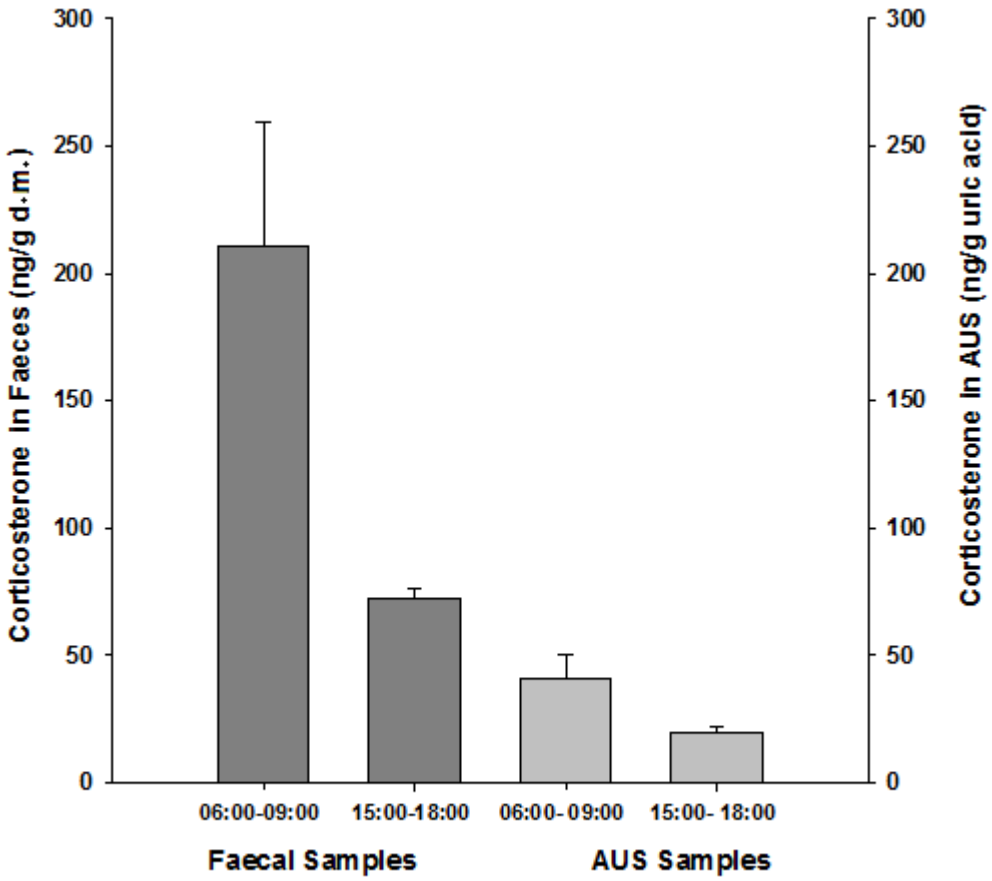


Figure 5.4. Corticosterone concentrations measured by ELISA in faeces (dark grey) using the Goymann et al., (2002) method and in avian urate spheres (light grey) from great tit (*Parus major*) guano, collected in the morning and late afternoon. Mean values with standard error bar.

Using the total guano mass and uric acid mass passed by each bird in each time period and the concentration of corticosterone in each sample (ng g^{-1} guano or uric acid), the total amount of corticosterone passed in the guano or AUS for each time period was calculated. There was no significant difference in guano total corticosterone (ng/3hrs) between the morning and late afternoon time periods ($p= 0.40$). The total amount of corticosterone excreted in the urine (ng/3hrs) during the morning and late afternoon was also not significantly difference ($p= 0.11$).

5.3.4. Diurnal changes in guano production

From drying and weighing the guano produced at three-hourly intervals by each individual great tit, the 24hr guano production of each bird was determined. Furthermore the uric acid content of each sample was determined as a measure of urine production, the dry mass of whole guano and uric acid are compared below (Fig 5.5).

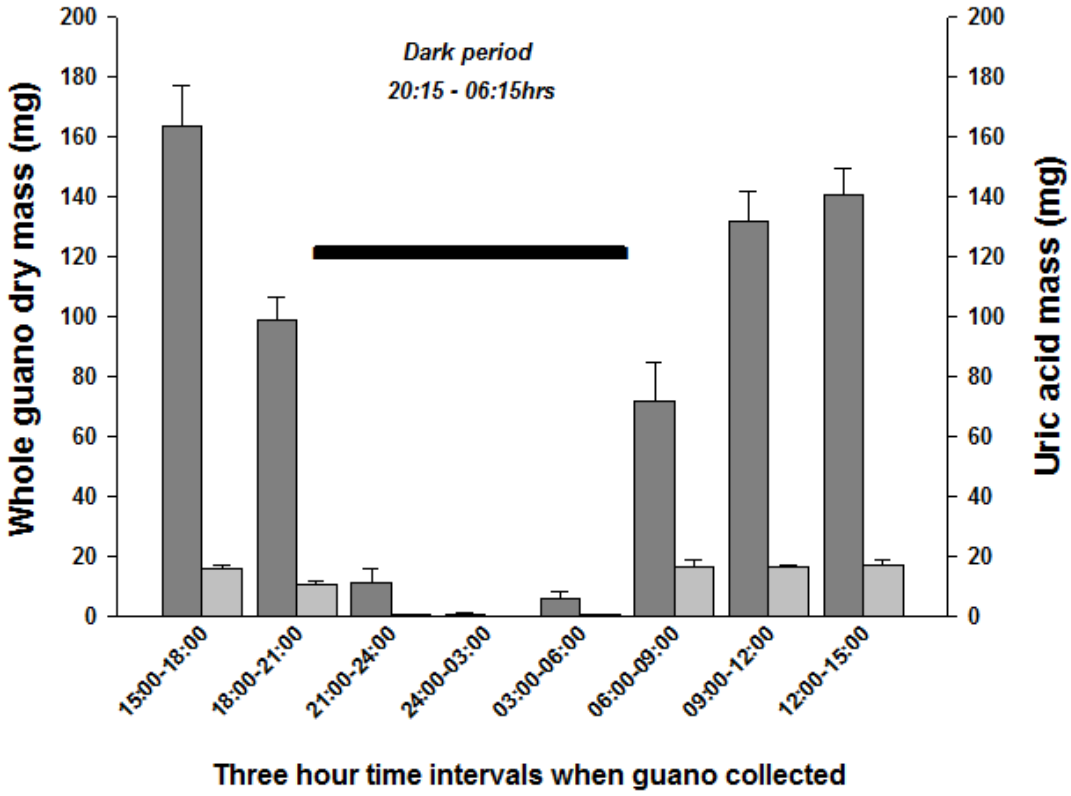


Figure 5.5. Total dry mass of guano (dark gey) and uric acid (light grey) passed by seven great tits (*Parus major*) in each 3 hour interval over the 24hr collection period. Mean values with standard error bar.

A one-way ANOVA of guano samples collected at different times in the light period showed whole guano dry mass differed significantly ($F(4,30) = 11.35$, $p < 0.001$). Tukey post-hoc pair wise comparison showed both the 9am and 9pm samples had significantly less mass than the 12am, 3pm and 6pm samples ($p < 0.05$). While there was no significant difference between the 9am and 9pm samples ($p = 0.176$) or between the 12am, 3pm and 6pm samples ($p = 0.927$, 0.170 and 0.427). A one-way ANOVA of urine production at different times in the light period showed uric acid content differed significantly ($F(4,24) = 12.99$, $p < 0.001$). Tukey post-hoc comparisons of the five light

period sample times indicate that the 9am, 12am, 3pm and 6pm samples (*M* = 14.99, 95% CI [12.69, 17.29], 14.97, 95% CI [11.52, 18.42], 14.59, 95% CI [11.10, 18.11] and 15.01, 95% CI [12.69, 17.29] respectively) all had significantly higher uric acid content than the 9pm samples (*M* = 10.67, 95% CI [5.78, 15.57]), $p < 0.05$. One-way ANOVA excluding the 6-9pm samples showed no significant difference in uric acid content between these light period sampling times ($F(3,18) = 0.30$, $p = 0.828$). However there was a significant difference between birds, in the amount of uric acid they each produced in these sampling times ($F(3,18 = 6.19$, $p = 0.001$).
These results show the lag in faeces production at the start of the light period from transit time (Clench and Mathias, 1992). In contrast urine production is not delayed in this way. However it appears that both faeces and urine production practically stop in the dark period.

5.3.5. Urine corticosterone concentrations following ACTH stimulation

The initial results from ACTH injections given to the great tits did not show the expected increase of corticosterone concentrations in AUS or blood samples. This was assumed to be a result of endogenous stress responses from handling and sampling, masking the effect of the ACTH (Wilson and Holberton, 2001). Consequently dexamethasone was given to the birds the day prior to ACTH injection in an attempt to resolve this problem. Plasma concentrations of corticosterone 40 min after an ACTH injection (Fig.5.6.) were significantly elevated over saline injected control birds ($t= 6.36$, $df= 5$, $p= 0.001$). The AUS corticosterone concentrations appeared to increase in both the saline and ACTH injected birds although neither were statistically significant ($p= 0.144$ and $p= 0.232$ respectively). Furthermore no significant difference was shown between treatments (Fig.5.7.).

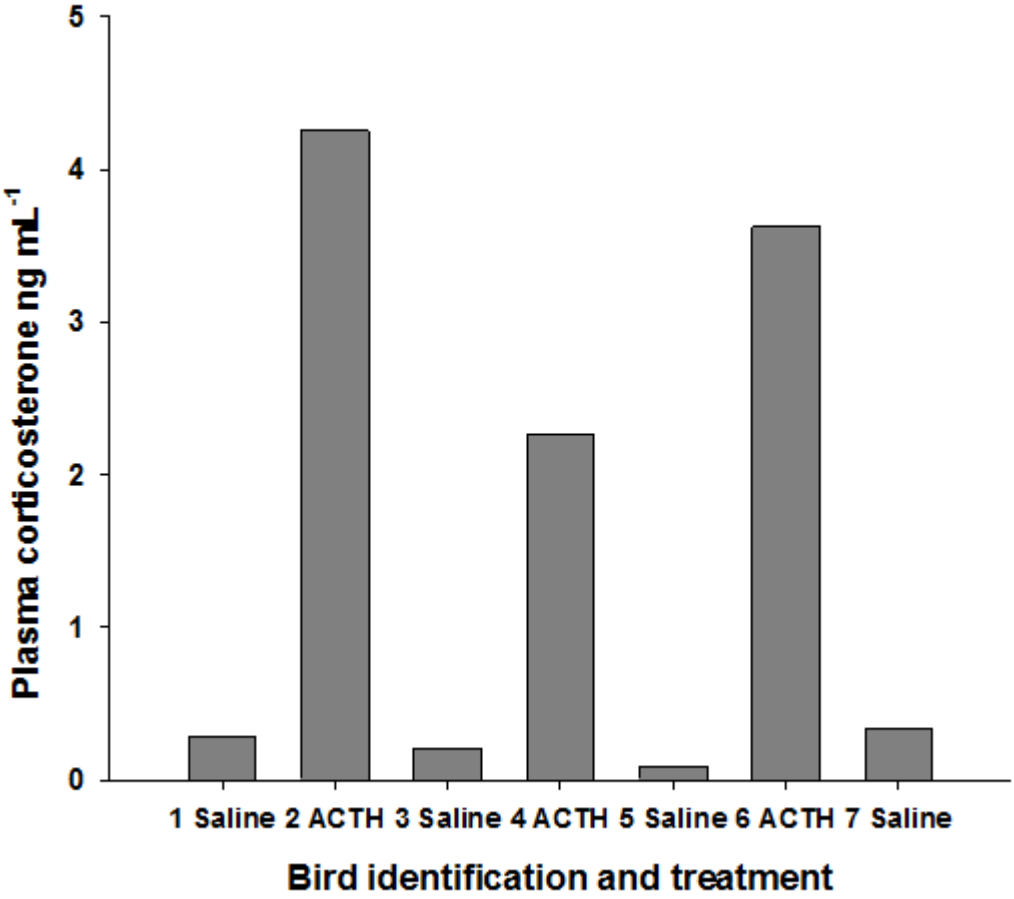


Figure 5.6. Plasma corticosterone concentrations in great tits (*Parus major*), measured by ELISA at 40 min after an IM injection of saline (control) or 1µg ACTH (Synacthen, Alliance), while under dexamethasone suppression. The limit of detection was determined to be 1.25ng mL^{-1} consequently all the saline treated birds had undetectable concentrations of plasma corticosterone.

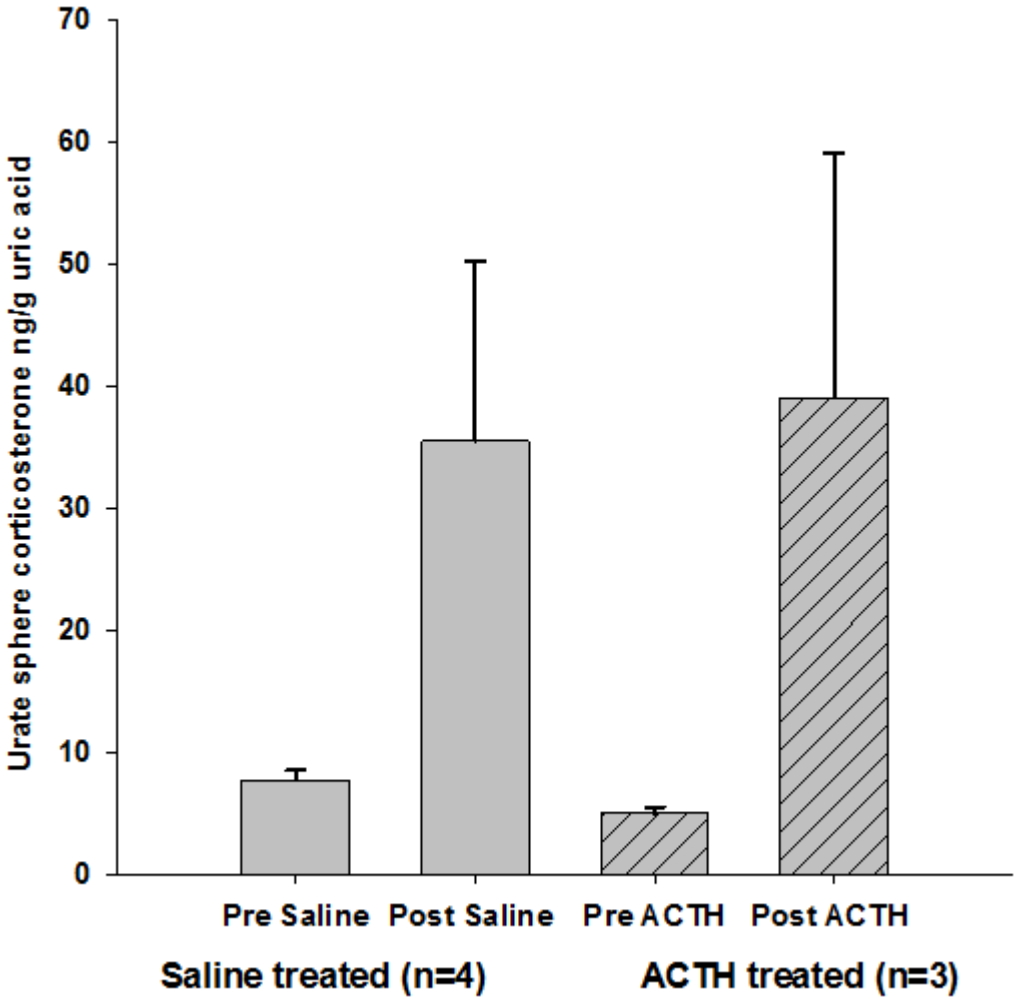


Figure 5.7. Great tit (*Parus major*) AUS corticosterone concentrations measured by ELISA before and after 1µg ACTH (Synacthen, Alliance) or saline (control) injections (given IM), while concurrently under the suppressive influence of dexamethasone. Mean values with standard error bar.

5.3.6. Dexamethasone suppression of corticosterone concentrations

The suppressive effect of dexamethasone on blood plasma corticosterone concentrations (mean 0.25ng/mL) was shown in four control (saline injected) great tits (Fig.5.6.), following an oral dose of 100µg of dexamethasone given the day before. Figure 5.8. shows corticosterone concentrations in AUS from all seven great tits were significantly reduced the day after receiving the same 100µg oral dexamethasone dose ($t= 4.58$, $df= 12$, $p= 0.001$). The low cross reactivity of dexamethasone with the ELISA antibody prevented its administration significantly elevating the measured corticosterone values.

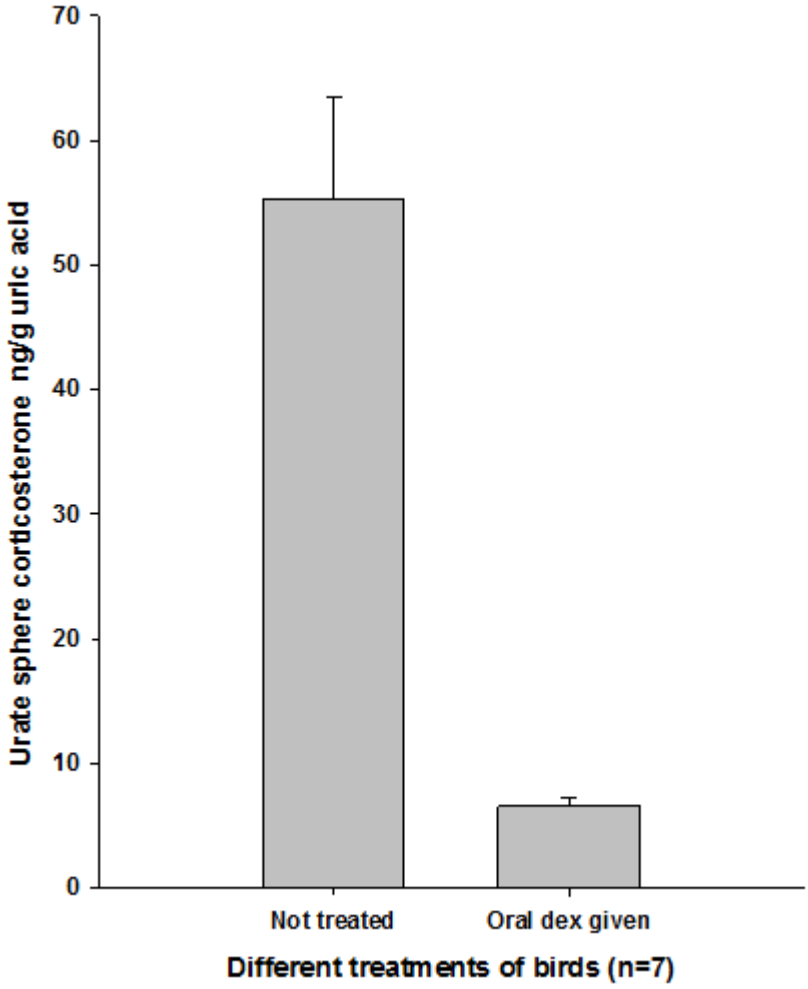


Figure 5.8. Great tit (*Parus major*) AUS corticosterone concentrations measured by ELISA before and the day after oral dexamethasone (100µg). Mean values with standard error bar.

5.3.6. Oral administration of corticosterone

Blood plasma corticosterone concentrations (Fig.5.9.) appeared elevated in the four birds given the 20µg oral corticosterone dose compared to the three placebo-dosed birds, however this was not statistically significant ($t = 1.90$, $df = 5$, $p = 0.116$). Although AUS corticosterone concentrations showed an apparent increase following the oral dose of 20µg corticosterone (Fig.5.10.), this was not statistically significant ($t = 2.19$, $df = 6$, $p = 0.071$).

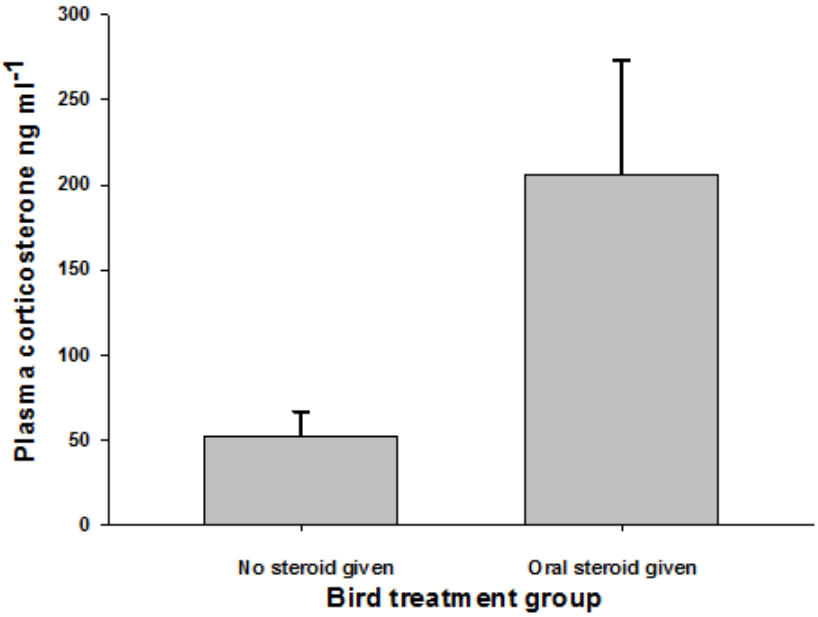


Figure 5.9. Comparison of great tit (*Parus major*) plasma corticosterone concentrations 20-30 min after the oral administration of placebo 20µL DMSO only (n=3) or 20µg of corticosterone in 20µL DMSO (n=4). Mean values with standard error bar.

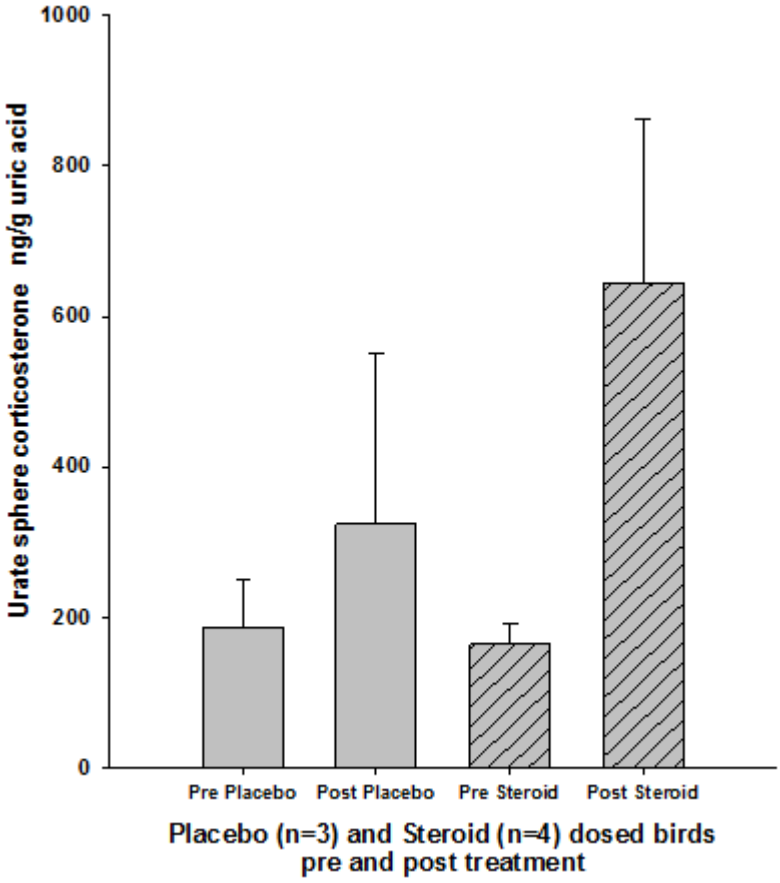


Figure 5.10. Great tit (*Parus major*) AUS corticosterone concentrations, measured by ELISA, before and after the oral administration to each bird of a placebo (solid column) or 20µg corticosterone (hatched column). Mean values with standard error bar.

5.3.7. RP-HPLC and LC-MS/MS detection of corticosterone in great tit AUS samples

Despite using solid phase extraction (SPE) to clean up the AUS extracts, no clear peaks attributable to parent corticosterone could be reliably identified using RP-HPLC. However *heart cut* samples eluting from the column between 10 and11 min (a time equivalent to the parent steroid) did contain parent corticosterone when analysed by LC-MS/MS (Table 5.3.). Furthermore analysis of the same *heart cut* samples by ELISA, detected the presence of a similar magnitude of corticosterone.

Table 5.3. LC-MS/MS and ELISA analysis for corticosterone of 1 minute heart cut samples from RP-HPLC separated great tit (*Parus major*) urate sphere samples (from guano collected following oral corticosterone administration) and a 200ng/mL corticosterone standard. The RP-HPLC data shows the variation of run time (min) of the nearest peak to parent corticosterone detected for each sample tested.

	RP-HPLC		**LC-MS/MS**	**ELISA**
Bird	**RT (min)**	**Area**	**ng/mL**	**ng/mL**
4	10.42	704	40	54
5	10.52	1192	50	50
6	10.34	1141	72	70
7	10.57	4288	2638	800
Standard	10.31	957	146	200

The *heart cut* analysis of the corticosterone standard (200ng/mL) using the ELISA method gave a recovery of 100% (Table 5.3.). However with the bird 7 result this should be treated as an estimate because the steroid concentration in the ELISA wells in both cases were above 15ng/mL, the reported upper limit of accuracy (IDS, 2007). The LC-MS/MS analysis represented a recovery rate of 73%.

5.4. Discussion

5.4.1. AUS extraction from guano

AUS were successfully separated from great tit guano using the alcohol suspension technique described. Furthermore it was shown that the residue from a currently used method of guano hormone analysis (Goymann et al., 2002) included intact urate spheres which on LC-MS/MS analysis contained parent corticosterone (Table 5.3.). This provides evidence that current techniques of guano steroid analysis (Goymann, 2005) discard some urine excreted parent corticosterone. However it is proposed that future work using a radio-labelled infusion study (Rettenbacher et al., 2004) should be carried out to resolve this issue.

5.4.2. Specificity of ELISA technique for measuring corticosterone in great tit AUS

Corticosterone concentrations in serial dilutions of AUS extracts from great tits showed approximate parallelism with the corticosterone standards suggesting specificity of the technique for measuring corticosterone in AUS. However faecal extracts using the method by Goymann et al., (2002), showed poor parallelism to standard corticosterone (Fig.5.1.), which may indicate interference from a matrix effect or metabolite cross reactivity (Goymann, 2005). In the light of this the results of faecal steroid analysis reported here may be questionable. An unusual feature of both plasma and AUS corticosterone concentrations in oral corticosterone protocol (see 5.3.6.) was their higher values compared with those reported in the literature and in the other analyses in this study. This may have been a consequence of the ELISA kit being defective, such higher values would equate with deterioration of the antibody possibly from improper storage.

5.4.3. The effect of gender on excreted corticosterone concentrations in great tits

In one experiment (Fig. 5.2.) AUS corticosterone concentrations appeared to confirm the reported finding that corticosterone excretion profiles from male and female birds are different (Rettenbacher et al., 2004; Goymann et al., 2002). However this difference was not shown in another experiment (Fig. 5.3.) where faecal and AUS corticosterone concentrations were measured concurrently. Despite this, as the birds throughout this

study were the same individuals (4 males and 3 females), different treatments to the whole group could be compared. In addition when the response of individual birds to a treatment was assessed (using before and after corticosterone concentrations), it was not necessary to take into account the sex difference.

5.4.4. Diurnal changes of corticosterone in great tit AUS extracts

The AUS corticosterone concentrations appeared to show a trend that confirmed a diurnal peak in the morning samples but it was not statistically significant (Fig. 5.4.). If a shorter time period for guano collection had been selected (e.g. 06:00 to 08:00hrs), it may have had a greater proportion of early morning urine and so may have shown the corticosterone peak, because of its reported rapid decline at dawn (Breuner et al., 1999). Although the total amount of corticosterone passed in guano over time is reported to be a better measure of a bird's hormone status than guano concentration (Goymann et al., 2006), no diurnal difference was shown using this method.

5.4.5. Diurnal changes in guano production

The quantity of guano produced by the great tits (Fig. 5.5.) in the first morning sample was less than later samples collected over the day, because of the transit time delay (Clench and Mathias, 1992) following the night fast. In contrast, the urine component of the guano, as measured by uric acid content, remains relatively constant over the day; suggesting a possible advantage of measuring steroid levels in AUS rather than in faeces. The constant urine production is understandable because it does not have the transit time delay inherent in faecal production. However it was noted the quantity of urine passed in the guano declined in the last collection period of the day which included a 45 minute period of darkness. Subsequent samples collected in total darkness produced dramatically less urine and faeces with many birds passing no guano at all. In all cases if guano was passed it did however contain some urine as evidenced by uric acid content. Although some authors have deduced urine formation is shut down at night as a consequence of a torpor state (Hartman-Bakken et al., 2004), a more likely explanation is that the majority if not all of the urine is refluxed into the lower bowel for re-assimilation (Laverty and Skadhauge, 2008).

5.4.6. Corticosterone response to ACTH administration

The significantly higher plasma concentrations of corticosterone following ACTH compared to saline injections in the great tits (Fig. 5.7.) confirm the birds were stimulated by the ACTH. The similarity in response to ACTH and saline injections as measured by AUS corticosterone concentrations (Fig. 5.8.) would suggest dexamethasone suppression of the HPA system was incomplete in these birds. Consequently endogenous stress responses were still able to contribute to the AUS corticosterone concentrations in the saline injected birds. However comparison between saline and ACTH treated birds is not valid in this experiment because of the possible gender variations in excretion profiles (see 5.4.3.).

5.4.7. Corticosterone response to oral dexamethasone

The low plasma corticosterone concentrations (mean 0.25ng/mL although below the detection limit of 1.25 ng/mL) following oral dexamethasone (Fig. 5.6.) was dramatically less than blood concentrations (mean 53ng/mL) in untreated great tits (Fig.5.10.) and below the basal concentration of 5.3 +/- 1.3ng/mL reported for great tits in the literature (Cockrem and Silverin, 2002). The significant reduction in AUS corticosterone concentrations following oral dexamethasone (Fig 5.8.) constitutes a *physiological* validation (Goymann, 2005) of this proposed technique to measure hormones in avian urine.

5.4.8. Corticosterone response to oral corticosterone

Both plasma (Fig. 5.10.) and AUS (Fig. 5.11.) samples showed an apparent but not statistically significant increase in corticosterone concentrations following oral corticosterone administration. The statistical power of this result was low because of the small sample size consequently more samples may have given a conclusive result. A further modification of this experiment would have been to pre-dose the birds with dexamethasone to reduce the endogenous stress response (Wilson and Holberton, 2001), which in this case may have obscured the effect of the oral corticosterone.

5.4.9. Comparing different analytical techniques for corticosterone detection

Hormone analysis of urine samples using liquid chromatography is recognised to be problematic because of the need for substantial pre-column clean up steps (M. Dunn, personal communication). Although the RP-HPLC method used could reliably identify parent corticosterone (standards), the additional constituents in AUS extracts obscured this steroid in sample analysis. This was in contrast to the clear traces achieved by other authors in the field, which suggests that future analysis should attempt to replicate their methods more precisely. One improvement to the method would be to use an internal standard such as dexamethasone which should resolve problems of peak identification and calibration (Wong et al., (1994). It was shown in this study the technique of taking *heart cut* samples constituted a further clean up step and allowed clear identification of the parent corticosterone by LC-MS/MS. The concurrent ELISA analysis of the timed *heart cut* samples detecting comparable concentrations of corticosterone was evidence the RP-HPLC column had separated the parent hormone. It was noted that earlier eluting *heart cut* samples (5-6 min) from AUS extracts also showed ELISA immuno-reactivity to corticosterone. This activity may equate to the more polar corticosterone metabolites which such ELISA techniques typically cross-react with (Goymann, 2005).

Although the presence of parent corticosterone was confirmed in AUS extracts by LC-MS/MS, validation was not possible due to the small number of samples analysed. Despite this, it is envisaged that future work will investigate the potential of replacing ELISA with LC-MS/MS analysis of the urate spheres. The reason being that ELISA methods are recognised to be inferior due to non-specific cross-reactivity, interference and matrix effects (Goymann, 2005). Furthermore LC-MS/MS is becoming the preferred alternative to immunoassays (ELISA and RIA) to quantify steroids in the clinical context (Carvalho et al., 2008; Soldin and Soldin, 2009). An added advantage is that LC-MS/MS analysis of a single sample can measure several steroids simultaneously (Hauser et al., 2008).

5.4.10. The utility of AUS corticosterone concentrations to assess avian stress

Comparing plasma and AUS corticosterone concentrations in great tits

These results showed that plasma and excreted corticosterone in AUS can be measured in great tits. The proposed technique using AUS to measure corticosterone status in

great tits was *physiologically* validated (Goymann, 2005) using oral dexamethasone. However the use of this drug can induce prolonged low corticosterone concentrations which are un-physiological, making it an unrealistic representation of normal steroid fluctuations in great tits.

Relatively short (< 1hour) increases in plasma corticosterone concentrations such as in response to ACTH or a single dose of exogenous corticosterone may have been hidden in the AUS by endogenous stress responses of the bird. In this respect detecting short or acute stress responses using AUS analysis would be problematic.

Sampling frequency and urine reflux

Great tits being small birds produce only small quantities of AUS, so the time required to collect adequate amounts of AUS for analysis is protracted. This reduces the resolution of measuring short lived changes in AUS corticosterone concentrations. The prolonged collection times (2-3hours) necessary in this study, may have contributed to the inability to detect brief changes in corticosterone concentrations. This sampling deficiency may be resolved using larger birds, facilitating more frequent AUS sampling. However the value of measuring short term stress responses may be questionable because long term stressors are more typically associated with the degradation of a bird's environment (Mormède et al., 2007).

A further restriction on AUS analysis is the process of urine reflux (Klasing, 2005), in which a proportion of the AUS are refluxed into the lower bowel and digested (Braun, 2009). Consequently the collectable AUS passed in guano only contain a partial and intermittent fraction of the total urine excreted corticosterone, so further hampering the detection of short lived changes.

Acute stress and coping styles

Research on chronic stress commonly contradicts the assumptions made from acute stress protocols that plasma concentrations of corticosterone equate to the severity of stress (Harvey et al., 1984). This is shown by chronically stressed birds having decreased basal concentrations of corticosterone (Cyr and Romero, 2007) and reduced HPA responsiveness (Rich and Romero, 2005). For this reason Mormède et al., (2007) warns against making firm conclusions on stress and hence assessment of welfare, from simply the measurement of circulating corticosterone concentrations.

A further complication when measuring acute stress responses is the effect of avian personalities or coping styles (Carere et al., 2003; Cockrem, 2005; Korte et al., 2005; Koolhaas, 2008). Coping styles are individually repeatable and can be bred for in birds (Carere et al., 2003). The significance of coping style is that it makes interpreting acute stress related corticosterone changes in free ranging birds (consequently with unknown coping styles) problematic.

Chronic stress causing diurnal flattening and dexamethasone resistance

Two reported biomarkers of chronic stress in diverse animal species are diurnal flattening and dexamethasone resistance (Touma et al., 2009). The former is shown by an elevation of the diurnal trough of basal plasma corticosterone concentrations. The latter is a relative unresponsiveness to the usually suppressive effect of dexamethasone on the HPA system (shown in this study). Although Carere et al., (2003) were unable to detect changes to diurnal concentrations of corticosterone in chronically stressed great tits this may have been because the stress was not severe enough. The degree of diurnal flattening depends on the severity and duration of the stress (Touma et al., 2009). Carere et al., (2003) also reported that two genetic lines of great tits with opposite coping styles showed similar diurnal activity. This may suggest that unlike acute stress responses which can vary with genetic coping style (Cockrem, 2005; Korte et al., 2005), chronic stress which causes functional changes to the HPA system, like diurnal flattening and dexamethasone resistance (Touma et al., 2009), could be more robust markers.

As the dynamic testing of the HPA system using dexamethasone, is advocated for animal welfare monitoring (Mormède et al., 2007) and chronic (persistent acting) stress is more relevant to environmental quality assessment (Mormède et al., 2007). The finding in this study that reduced corticosterone concentrations from oral dexamethasone are measurable in AUS, may suggest dexamethasone resistance could be detectable in chronically stressed birds using the same technique. Consequently the measurement of corticosterone concentrations in AUS following orally administered dexamethasone, although restricted to captive birds, could be a potential biomonitor for avian welfare and environmental quality assessment.

Catecholamines as future avian stress biomarkers

An alternative to measuring corticosterone concentrations to assess environmental stress in birds may be to measure catecholamine concentrations in their AUS. Catecholamines, principally dopamine, adrenaline and noradrenalin, are very important mediators of the stress response. Furthermore the activation of the sympatho-adrenomedullary (SAM) system is among the earliest responses to stress (Sapolsky, 2002). Advantages of assessing the SAM over the HPA system include catecholamine activity precedes corticosteroid activity and it does not appear to be as modulated (Spasojevic et al., 2009). As 15% of circulating catecholamines are strongly protein bound (mainly to albumin) in plasma (El-Bahr et al., 2006), this affinity may also occur with the albumin in AUS (Janes and Braun, 1997). In addition a recent study in chickens has shown that the urinary excretion of catecholamines is the main route of their elimination (Lepschy et al., 2008). Consequently it is expected that catecholamines could be measured in extracts of AUS by a similar LC-MS/MS method used to detect catecholamines in human urine samples (Whiting, 2009).

5.5. Conclusions

This study showed that the hormone corticosterone was detectable in extracts from AUS separated from avian guano. This met the first aim of the study to develop a method to extract and analyse this hormone from AUS. Furthermore this represents urinary excreted corticosterone discarded by current techniques which analyse this hormone in guano (Goymann, 2005). The second aim of the present study, to validate the AUS method, was accomplished to some extent by detecting the suppressive effect of dexamethasone on corticosterone levels, measured by ELISA, in AUS extracts. However other findings left many questions unanswered in the search for a non-invasive method to monitor stress in birds, which may in part be a consequence of our limited knowledge of the avian urinary system.

Chapter 6.

General discussion

6.1. Separating AUS from the faeces in avian guano samples

In birds, urine is passed with faeces, with a variable amount of the urine being incorporated in faeces from urine refluxed into the lower bowel (Braun, 2009). Urine reflux is a post renal modification conservation strategy with the proportion of urine refluxed depending upon the bird's state of hydration and diet (Laverty and Skadhauge, 2008). When urine is refluxed the constituent AUS are disrupted and the liberated uric acid is broken down by microbial uricase enzymes (Braun, 2009). The intact AUS in guano represent a fresh non-digested component of avian urine, which can be extracted as a stable suspension in alcohol (Drees and Manu, 1996). After drying, these alcohol extracted AUS constitute a stable powder suitable for storage and chemical analysis. Only by stressful catheterisation (Styles and Phalen, 1998) can all the excreted urine be collected, the proportion of excreted urine collected non-invasively from guano deposits will depend upon how much is refluxed (Laverty and Skadhauge, 2008). If a large proportion of urine is refluxed into the lower bowel, urinalysis from guano deposits may not detect short lived changes in analyte concentrations (see ACTH response Chapter 5). However prolonged changes as suggested in heavy metal exposure (see Chapter 3) and persistent hormone concentrations (see dexamethasone suppression in Chapter 5) are detectable.

6.2. Quantification of AUS constituents using uric acid analysis

Because the AUS of diverse species are consistently 65% uric acid by dry mass (Casotti and Braun, 2004), contaminants present in AUS can be quantified against uric acid concentrations. The accuracy of uric acid analysis is sensitive to the quantity of sample being analysed because uric acid is poorly soluble even in caustic solutions. For this reason high sample mass may give reduced extraction efficiencies and so falsely low uric acid concentrations. It appears that uricase is highly active in the guano of birds as the faecal component of guano has little uric acid content despite the addition of refluxed urine (Braun, 2009). This fact is not surprising as many bacteria and fungi

possess uricase activity (Yazdi et al., 2006) and such organisms are abundant in the avian digestive system (Klassing, 2005). A benefit of using alcohol for urine extraction is that it kills such organisms and possibly denatures the uricase enzyme, preventing uric acid destruction.

6.3. Heavy metals in AUS

In Chapter 3 chickens with access to heavy metal contaminated soil showed elevated concentrations of Pb, Cu and Zn in their AUS compared to the AUS from control birds and the same birds following soil remediation. The prolonged excretion of Pb, assumed to be from sequestered bone deposits (Scheuhammer, 1996), after site remediation gave proof that this metal was being detected in the urine rather than simply resulting from faecal contamination of the AUS samples. However this study was seriously limited in its design as it lacked a suitable control and had an inadequate number of independent samples, which precluded statistical analysis.

In Chapter 4 the low metal concentrations detected in seabird AUS agreed with the lack of metal contamination reflected in concurrently collected tissue samples and in the re-analysed fish which constitute the diet of these seabirds. As metal concentrations were detectable in the AUS under these conditions, it suggests any increase in the seabirds' exposure to bioavailable environmental metals, should readily be detected using this method of urinalysis. The findings of this study were also limited by its design. If direct sampling of the nest-bound chicks had been used this would have provided more robust evidence for the use of AUS to monitor the seabirds' metal exposure.

6.4. Hormones in AUS

The stress hormone corticosterone was detected by ELISA in AUS extracts. Parent corticosterone was identified in extracts of AUS using LC-MS/MS, although this method of analysis did not provide evidence of hormone changes in the birds. Short changes in plasma corticosterone concentrations were not consistently detected in AUS using ELISA, possibly on account of endogenous stress responses (Wilson and Holberton, 2001) and also urine reflux (Laverty and Skadhauge, 2008) preventing the analysis of some of the excreted urine. However the more prolonged change in plasma

corticosterone from dexamethasone suppression was reflected by ELISA detected corticosterone concentrations in AUS samples, constituting a physiological validation of this method (Goymann, 2005). It is concluded that infusion experiments using radio-labelled corticosterone should be performed to resolve the clear discrepancy between this hypothesis and current literature, which implies that it is very unlikely that parent hormone is present in urine (Rettenbacher et al., 2004) or furthermore AUS (Wasser and Hunt, 2005).

6.5. Concluding remarks and future research

Despite the many short comings in experimental design, the present study has shown that potentially relevant concentrations of metals and corticosterone can be measured in AUS.

AUS have been shown to exhibit robust physical properties making them suitable for collection, storage and analysis from guano of both wild and domestic birds.
From this initial investigation future research into the use of AUS for urinalysis and as a non-invasive biomonitoring method is envisaged to follow three paths.
1. To determine the precise mechanism of AUS formation in birds including its genetic control. This would allow a deeper understanding of how and why biologically relevant substances may be incorporated within AUS.
2. To use more precise and sensitive analytical methods such as the LC-MS/MS analysis of AUS extracts to specifically identify biomarkers of a bird's physiological state and environmental exposure.
3. To develop methods for analysing the protein constituents of AUS. This could include the identification of carrier proteins such as metallothioneins and CBG, disease specific protein abnormalities, and proteins to allow individual finger printing.

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