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LEAD EXPOSURE IN FREE-RANGING KEA (*NESTOR NOTABILIS)*, TAKAHE (*PORPHYRIO HOCHSTETTERI)* AND AUSTRALASIAN HARRIERS (*CIRCUS APPROXIMANS)* IN NEW ZEALAND

A thesis presented in partial fulfillment of the requirements for the degree of

Masters of Veterinary Science

in

Wildlife Health

at Massey University, Palmerston North,

New Zealand.

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2009

Abstract

Lead is a highly toxic metal that has been used by humans for over 2000 years. Over this time it has become increasingly apparent that despite its usefulness, lead is one of the most highly toxic substances known to man. Current research into lead exposure of humans focuses on low-level chronic exposure and its effects on learning and behaviour. However, investigations into lead exposure of wildlife are still focussed on mortalities, particularly of waterfowl and raptors, with little known about low-level exposures or the effects on other species.

This study examines the exposure of free-ranging kea (*Nestor notabilis*) from the Aoraki/ Mt Cook village and national park, takahe *(Porphyrio hochstetteri)* from Tiritiri Matangi, Kapiti and Mana Islands, and the lead associated syndrome of clenched-claw paralysis and leg paresis in harriers (*Circus approximans*) in New Zealand.

Thirty-eight kea had detectable blood lead with concentrations ranging from 0.028 mg/L to 3.43 mg/L (mean = 0.428 mg/L ± 0.581). Analysis of tissue samples found that seven of 15 birds died with elevated tissue lead. Lead exposure may be an important contributing factor in kea mortality. As a result of these findings, lead abatement in areas frequented by kea is being considered.

Eighteen of 45 takahe had detectable blood lead concentrations ranging from 0.015 mg/L to 0.148 mg/L (mean = 0.028 mg/L ± 0.042). Analysis of tissue samples from offshore island and Murchison Mountains birds found that all had detectable lead. Despite levels of lead exposure in the population being low and unlikely to result in overt clinical signs, it is widespread and there may be significant exposure of birds living around old buildings.

An investigation into the clinical signs, pathology and response to treatment of clenched-claw paralysis and leg paresis in wild harriers was carried out. Harriers with clenched feet had significantly higher blood lead concentrations than those without. In conclusion, lead is a major factor in the expression of this clinical syndrome but other factors not yet identified are playing a role.

This study demonstrates that lead is widespread in the New Zealand environment exposing a diverse range of avifauna, and has made some progress towards exploring some of its effects on health and survival.

Acknowledgements

The research presented in this thesis was very much a collaborative effort and could not have been achieved without the advice and assistance of many people. Firstly, thanks must go to my fabulous supervisors, Dr Brett Gartrell and Dr Wendi Roe. Their friendship and patience during the two years of my residency (and subsequent writing period!) was more than a student could have asked for. The many coffees and chats shared not only helped with getting through the research and clinical work, but also the difficulties of an international long-distance relationship.

Thanks must also go to Dr Kerri Morgan, my fellow resident for the first few months and subsequent fellow wildlife first-aider, who has became a great friend and confidant. Many late nights were spent studying for membership exams, writing powerpoints, walking for charity and solving the problems of our world.

Thankyou to Bridey White, one of the best wildlife technicians I have ever worked with as well as a great friend, who helped keep me sane during periods of exhaustion, always free for a coffee and a chat or a dog walk with Boston. I miss you BJ!

Thanks to my wonderful Mt Cook field buddies and friends, Dr Kate McInnes and Clio Reid. Their knowledge and assistance in the field with catching and sampling the cheeky kea was invaluable and I couldn't have completed my studies without them. Kate, thank you also for your support through the Department of Conservation in so many ways.

A big thank you to the volunteers who headed out into the field with me, especially Bridget Wrenn, Andrew Hill, Karen Lithgow and Josie. You all helped to make the field work go smoothly and be even more fun.

To the Department of Conservation (DOC) staff from the Aoraki/Mt Cook area office, Mana, Kapiti and Tiritiri Matangi islands and Wellington – thank you for all your support, particularly in the field. The Takahe Recovery Group, especially Jane Maxwell for your assistance with obtaining the tissue samples; Gyula Gajdon (Jussy) for sample collection; the Canterbury Mountaineering Club and DOC for field accommodation; the University of Vienna Kea Research Project group for field equipment; Felicity Jackson and the staff at the Massey University Institute of Food, Nutrition and Human Health Laboratory for tissue lead analysis; Drs Craig Johnson and

Joe Mayhew for assistance with the electroneurophysiology and for looking over the histopathology with me.

Evelyn, Pat and Mary from the IVABS histopathology lab – thanks for processing all my samples with a smile – even when large numbers arrived late on a Friday afternoon

Thanks to Dawn Morton, wildlife rehabilitator extraordinaire – your passion and dedication is inspirational; to the staff and volunteers at the New Zealand Wildlife Health Centre and fellow residents Vanessa Gray and Andrew Hill; to all the staff at IVABs and my flatmates Jamie, Tracey and Paul for being a big extended family, and to my family for support from afar.

This research was carried out with permission from the Takahe Recovery Team, DOC Canterbury Conservancy office and Ngai Tahu. The Massey University Animal Ethics Committee (MUAEC) provided ethics approval (protocol 06/22). Funding for this work was provided in part by an IVABs, Massey University post-graduate research grant, an IVABS Avian Health Fund grant as well as financial and in-kind support from the New Zealand Department of Conservation.

Last but by no means least, thanks must go to my ever-enduring husband David McLelland for his love, support, advice and patience with a wife who has an inability to stay in one place for too long.

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Abbreviations Used in Text

A/G ratio	Albumin:globulin ratio
AChE	Acetylcholinesterase
ALAD	aminolevulinic acid dehydatase
AST	Aspartate Aminotransferase
ASV	Anodic stripping voltammetry
BBB	Blood Brain Barrier
BUN	Blood Urea Nitrogen
CDC	United States Center for Disease Control
CK	Creatine Kinase
CNS	Central Nervous System
EP	Erythrocyte protoporphyrin
GGT	γ-glutamyl Transferase
H&E	Haematoxylin and Eosin
ICP-MS	Inductively coupled plasma mass spectrometry
ISIS	International Species Information System
IUCN	World Conservation Union
LH	Lutenising hormone
MCV	Motor Conduction Velocity
MUVTH	Massey University Veterinary Teaching Hospital
NZ	New Zealand
NZWHC	New Zealand Wildlife Health Centre
Pb	Lead
PbS	Lead Sulphide (galena)
PCV	Packed Cell Volume
PNS	Peripheral nervous system
RBC	Red blood cell
s.d.	Standard deviation
s.e.	Standard error
T ½	Half life
WCC	White Cell Count
WHO	World Health Organisation
ZN	Ziehl-Neelson

Chapter 1

Introduction, Literature Review and Objectives

"*You will see by it, that the Opinion of this mischievous Effect from Lead is at least above Sixty Years old; and you will observe with Concern how long a useful Truth may be known and exist, before it is generally receiv'd and practis'd on*"
Benjamin Franklin, in a letter to Benjamin Vaughan, July 31, 1786

1.0 Introduction

Lead toxicity is a serious and commonly encountered problem in both wild and captive animals and humans around the world. To date, research into lead exposure and toxicities in wild animals has focussed on waterfowl, raptors and upland game birds with very little information reported for other species. Investigations into the effects of lead exposure in humans has begun to focus more on the sub-clinical and developmental effects of low-level and chronic exposure, particularly on the nervous system, focussing on learning and behavioural changes. Conversely, research into the effects of lead exposure on wild animals still focuses on mortality events, usually related to point sources of high lead contamination, or the effects on individuals of endangered species such as bald eagles (*Haliaeetus leucocephalus*). Lead exposure not causing debilitation or mortality is generally assumed to be insignificant.

This chapter presents a review of lead's properties, use and distribution; the pathogenesis of toxicity; clinical and sub-clinical effects on humans and wildlife; an overview of lead in the environment; assessment of lead exposure and summarises the current information regarding lead in the New Zealand environment and exposure of New Zealand wildlife.

2.0 History of Lead

Lead is a highly toxic non-essential heavy metal that is found throughout the environment. Lead is found naturally in small quantities in limited areas; however anthropogenic use of lead, especially since the industrial revolution of the 1850s, has caused environmental contamination significant enough to affect free-ranging wildlife (Hoffman, 2003). Lead is one of the most toxic metals known due to its wide ranging effects on multiple body systems (Pattee and Pain, 2003). Morbidity and mortality in animals (including humans) as a result of lead poisoning has been recognised for more than 2500 years (Locke and Thomas, 1996) with clinical descriptions of lead intoxication first recorded by Greek physicians in 100BC (Gilbert and Weiss, 2006). Despite its toxicity, lead is an extremely useful substance and has been used extensively by many civilisations throughout time. The use of lead as a medicinal agent was practiced in many cultures (Pueschel et al., 1995). Ancient Egyptians and Romans utilised lead as a sweetener in wine, to line cooking vessels and in the construction of

items such as water pipes, coins and cosmetics, and some suggest that lead poisoning may have contributed to the downfall of these civilisations (Locke and Thomas, 1996; Pattee and Pain, 2003; Needleman, 2004).

Pattee and Pain (2003) note that despite over 7000 years of use, the local and global availability and distribution of lead has been altered more than that of any other toxic element during the last 300 years and particularly in the last century. Studies of lead isotopes in human tissues have shown that pre-industrial humans had lead levels approximately 500 times lower than post-industrial humans (New Zealand Commission for the Environment, 1986). Today, while no longer commonly used in products for food and drink preparation or consumption, lead is still used in household items such as construction materials, curtain weights, ammunition, batteries and paint (Locke and Thomas, 1996; Gwaltney-Brant, 2002), and in some countries it is used in makeup (Parry and Eaton, 1991), lolly wrappers, jewellery and toys (CDC, 2007a,b,c).

Up until recently, lead poisoning in humans was considered primarily an occupational hazard for adult humans (Pueschel et al., 1995). In the 20th century, lead poisoning in children was recognised as a distinct disease entity and over the past 100 years, research into lead poisoning has seen the sub-clinical and chronic effects of lead acknowledged (Pueschel et al., 1995). This research has also resulted in laws to decrease exposure and reductions in the acceptable "normal" blood lead concentrations (Table 1.1) in humans from 0.8 mg/L in adults and 0.6 mg/L in children in the late 1960s to 0.25 mg/L in non-occupationally exposed adults and 0.1 mg/L in children by 1991 in the USA (Pueschel et al., 1995) and 0.15 mg/L for all groups in New Zealand (Ministry of Health, 1998).

In wild animals, lead is considered to be primarily a problem of waterfowl and birds of prey. In wild birds, lead toxicosis is the most commonly reported heavy metal toxicosis and over the last 112 years there has been extensive reporting of lead poisoning in North America, Europe, Scandinavia and Australia, usually attributed to lead shot (Samour and

Table 1.1 Decreases in blood lead minimum action levels in humans between 1960 and 2007

Year	Country	Age	Reference value (mg/L)	Reference
1960	USA	Adult	0.8	1
1960	USA	Children	0.6	1,2
1991	USA	Adult	0.25	1, 3
1991	USA	Children	0.1	1, 2
1993	Australia	All	0.1	4
1995	World	Adult	0.25	5
1995	World	Children	0.1	5
1998	New Zealand	All	0.15	6
2004	USA	Lead workers	0.4	7
2005	Australia	All Australians	No limit set	8
2006	World	Children	0.02 (proposed)	2
2007	USA	All	No limit set	9

1. Pueschel *et al* (1995); 2. Gilbert and Weiss (2006); 3.Lauwerys and Hoet (2001); 4. National Health and Medical Research Council (1993) 5.WHO (1995); 6. Ministry of Health (1998); 7. Gidlow (2004); 8. National Health and Medical Research Council (2005); 9 Agency for Toxic Substances and Disease Registry (ATSDR) (2007)

Naldo, 2005). Other avian wildlife reported with lead poisoning include kea (*Nestor notabilis)* (Jarrett, 1997) Laysan albatross (*Diomedea ummutablis)* (Sileo and Fefer, 1987), and passerines (Fisher et al., 2006; DEC, 2007).

3.0 Physical and Chemical Properties of Lead

Lead (Pb) is a soft, blue/grey, heavy metal element with the atomic number 82 and an atomic weight of 207.2 Daltons. There are four isotopes, ^{204}Pb ^{206}Pb, ^{207}Pb and ^{208}Pb found in nature (Dart et al., 2004). It is a very easily mined metal and makes up around 0.0013% of the earth's crust (Locke and Thomas, 1996). The most common form in nature is the lead sulphide 'galena' (PbS) (Locke and Thomas, 1996). Lead forms many different compounds which have useful properties (Table 1.2). Lead's usefulness arises from its soft, malleable and corrosion resistant properties. It has a very high density making it useful for blocking out harmful radiation. Most lead compounds

are relatively insoluble in water, however, the solubility increases in acidic environments such as a bird's ventriculus (Locke and Thomas, 1996).

The potential for toxicity depends on the chemical and physical form of the lead compound and the route of exposure (Pattee and Pain, 2003). Solubility and particle size influences adsorption into sediments and absorption by living organisms (Pattee and Pain, 2003). Lead exists in organic and inorganic forms. The most common source of organic lead is petrol products. The alkyl form (e.g. tetraethyl lead) is lipid soluble and consequently can be absorbed through the skin in addition to other exposure routes, and is highly neurotoxic (Yasutake and Hirayama, 2002). The inorganic lead salts, for example, lead chromate and lead acetate are found in lead based paints (Anderson et al., 1996). Due to its high solubility lead acetate is highly toxic when ingested, however, it is believed to be a lower risk for pulmonary intoxication as it does not easily form dusts (Anderson et al., 1996).

Table 1.2 Lead compounds and examples of their uses.

Lead Compound	Chemical Formula	Uses
Lead metal	Pb	Tank and pipe lining; solder; protective equipment X-rays.
Lead acetate	$Pb(C_2H_3O_2)_2$	Mordant (setting agent) in cotton dyes; lead coating of metals; pant, varnish and ink drier; astringent in medicinal
Lead arsenate	$Pb_3(AsO_4)_2$	Insecticide and anti-cestode medication
Lead azide	$Pb(N_3)_2$	Primers and explosives.
Lead carbonate	$2PbCO_3Pb(OH)_2$	White lead
Lead chloride	$PbCl_2$	Lead salts, lead chromate pigments, analytical reagent, solder and flux in printed circuit boards
Lead chromate	$PbCrO_4$	Yellow pigment
Lead dioxide	PbO_2	Electrodes in lead-acid batteries, matches, explosives, curing agent for polysulphide elastomers.
Lead Fluoborate	PbB_2F_8	Material finishing operations.
Lead Fluoride	PbF_2	Chemical manufacture, marine paint, electronic and optical parts, high-temp dry-film lubricants; special grades of glass
Lead iodide	$Pb I_2$	Bronzing, gold pencils, mosaic gold, printing and photography.
Lead molybdate	$PbMoO_4$	
Lead monoxide	PbO	
Lead nitrate	$Pb(NO_3)_2$	
Lead oxide	Pb_3O_4	Red pigment, rust proofer and steel primer.
Lead oxychloride	$PbCl_2.2PbO$	
Lead peroxide	PbO_2	
Lead phosphate	$Pb_3P_3O_8$	Stabiliser in styrene and casein plastics.
Lead sesquioxide	Pb_2O_3	
Lead silicate	$PbSiO_3$	
Lead stearate	$Pb(C_{18}H_{35}O_2)_2$	Extreme pressure lubricant and dryer in varnishes.
Lead subacetate	$Pb_3C_4H_{10}O_8$	Decolourising agent in sugar solutions; analytical chemical
Lead suboxide	Pb_2O	
Lead sulphate	$PbSO_4$	Storage batteries, paint pigments, alloys, fast drying oil varnishes, fabric weights and lithography.
Lead sulphide	PbS	Ceramics, infrared radiation detectors and semiconductors.
Lead thiocyanate	$Pb(SCN)_2$	Safety matches, primers in arms cartridges, pyrotechnics, dyes.
Tetraethyl lead	$Pb(C_2H_5)_4$	Organic: Anti-knock agent in leaded petrol
Tetramethyl lead	$Pb(CH_3)_4$	Organic: Anti-knock agent in leaded petrol

Table modified from Dart, Hurlbert and Boyer-Hassen (2004) and Pohanish (2002)

4.0 Pathogenesis of Lead Toxicity

4.1 Overview of Lead Toxicity

The renal, gastrointestinal, reproductive, central nervous and peripheral nervous systems and the biosynthesis of haeme are all affected by inorganic lead (Verity, 1997). Exposure duration of toxins can be described as acute (short exposure or single dose), subacute (exposure lasting a few days), subchronic (a few weeks to a month of exposure) or chronic (an organism is exposed for a considerable part of its lifespan) (Fairbrother, 1999). This terminology and definitions are slightly different to more familiar terms used in pathology where peracute (minutes to hours), acute (hours to three -five days), subacute (three-five days to seven-14 days) and chronic (seven -14 days to weeks, months or years) are used (Bochsler and Slauson, 2002).

4.2 Lead Biokinetics

There is inter- and intra-species variability in the uptake, distribution, toxic effects and elimination of lead (Silbergeld and Goldberg, 1980; Pattee and Pain, 2003). These differences lead to a wide range of responses to lead exposure (Pattee and Pain, 2003).

4.2.1 Intake and absorption

The uptake of lead into an individual depends on the concentration in the environment, the chemical and physical form, the route of exposure and the species being exposed (Pattee and Pain, 2003). Within species; age, sex, and nutritional status may affect the uptake of lead (Lauwerys and Hoet, 2001). Variable mortality and tissue lead levels were seen in mourning doves in controlled environments that were experimentally dosed with a uniform amount of lead suggesting that there is marked individual biological variation in uptake and absorption (Kendall et al., 1996).

Inorganic lead is usually ingested and/or inhaled by non-aquatic animals, and organic lead (e.g. tetraalkyl lead) can be ingested, inhaled or absorbed through the skin (Pattee and Pain, 2003). Differences between species in lead absorption, distribution in body tissues, detoxification and excretion lead to a wide range of responses to lead exposure (Pattee and Pain, 2003). Approximately two percent of ingested lead is absorbed through the intestine in cattle (*Bos taurus)* and sheep (*Ovis ovis)* (Summers et al., 1995; Pattee and Pain, 2003) however Pattee and Pain (2003) reviewed several

studies and found that ten percent (range eight to 18 %) is most frequently reported for adult humans. An uptake of up to 50 % of dietary lead is reported for children (WHO, 1995; Anderson et al., 1996; Berney, 1996; Gwaltney-Brant, 2002). It is likely that uptake in avian species is as variable as in mammals, however it is possible that birds with muscular gizzards for grinding food may experience increased uptake as lead pellets may remain in the gizzard for approximately six weeks until they are completely broken down and absorbed (Pain, 1996). Intestinal absorption is also dependent on dietary factors resulting in the large variations of percentage absorption reported in the literature (Pattee and Pain, 2003). Most food reduces lead absorption however high fat diets may increase absorption (Anderson et al., 1996). The amount of certain dietary elements can inhibit or promote uptake and toxicity of lead, however, interactions between dietary components and lead are very complex and excesses of the same compounds may also intensify lead toxicosis (Lauwerys and Hoet, 2001; Pattee and Pain, 2003). Dietary calcium and iron affect intestinal lead absorption and toxicity with low calcium or low iron diets increasing absorption, toxicity and storage in soft tissues (Anderson et al., 1996; Pattee and Pain, 2003). However, Anderson et al (1996) comments that lead may be competitively inhibited by a diet containing sufficient essential minerals.

Lead can be ingested with food or water, or on its own (Pattee and Pain, 2003). For example, lead shot may be ingested by waterfowl as a grit substitute and kea may ingest lead when performing destructive behaviours. Lead is also known to be sweet tasting (Locke and Thomas, 1996) and some animals may deliberately ingest it.

Most lead deposited into the lungs following inhalation is absorbed into the bloodstream (Pattee and Pain, 2003), although this is dependent on the size of the particles and to some extent the type of lead (Anderson et al., 1996). In adult humans approximately 50% of inhaled lead particles less than 5 µm diameter are absorbed, however studies in rats (*Rattus rattus)* have shown 90-98 % absorption of these smaller particles (WHO, 1995). Larger particles deposit in the upper respiratory tract and are moved up by the mucociliary apparatus and ingested (WHO, 1995; Gwaltney-Brant, 2002).

Lead embedded in tissue is poorly absorbed and is not considered a significant route of exposure due to the poor bioavailability of non-ionised lead (Gwaltney-Brant, 2002). Guillemain et al (2007) found during a mark-recapture study of nearly 40,000

teal (*Anas crecca)* that birds with embedded lead shot (diagnosed using radiography) have lifespans equivalent to birds with no embedded shot.

4.2.2 Distribution

Lead kinetics are usually examined using a compartmental model consisting of blood, soft-tissue and bone (Dart et al., 2004). In this model, there are three main compartments of lead distribution, the first consisting of the bloodstream and some rapidly exchanging soft tissue with a half life of approximately 35 days in adult humans (Anderson et al., 1996; Lauwerys and Hoet, 2001). The remaining soft tissues are the second compartment with a half life of approximately 40 days in adult humans (Anderson et al., 1996; Lauwerys and Hoet, 2001). As different tissues have different turnover times, this is an average half-life. The third component is bone with half lives ranging from less than ten to greater than 15 years in humans depending on the bone type with medullary bone turning over faster than cortical bone (Lauwerys and Hoet, 2001). Peer-reviewed publications on the half-life (T½) of lead in avian tissues were unable to be found, however an estimated T½ of lead in blood of approximately 13 days has been determined for Californian condors (*Gympnogyps californianus)* naturally exposed to lead (Fry, 2003). T ½ of blood lead in turkey vultures (*Cathartes aura)* and red tailed hawks (*Buteo jamaicensis)* is estimated to be seven and 14 days respectively following an experimental study involving one turkey vulture and two red-tailed hawks (Reiser and Temple, 1980).

Following uptake, lead enters the bloodstream and is carried around the body attached to the surface of red blood cells (Anderson et al., 1996; Lauwerys and Hoet, 2001; Pattee and Pain, 2003). Within minutes the majority of the lead is deposited in soft tissues (Pattee and Pain, 2003). Over half of the absorbed lead is eventually deposited in the bones (approximately 90% in humans (Lauwerys and Hoet, 2001) and around a quarter is deposited in the kidneys (Summers et al., 1995). In the proximal convoluted tubular cells of the kidney and occasionally in hepatocytes, lead is deposited in protein complexes within the nuclei, forming acid-fast inclusions (Pattee and Pain, 2003). Pattee and Pain (2003) suggest that these complexes may reduce cellular toxicity. Renal tubular cell intra-nuclear inclusions associated with lead have been identified in a wide range of avian and mammalian species (Pattee and Pain, 2003).

Tissue distribution also varies with acute or chronic exposure. Acute exposure results in higher soft tissue levels while chronic exposure results in higher bone levels.

Generally bone, teeth, liver, lung, kidney, brain and spleen have been found to contain the highest concentrations of lead (Gwaltney-Brant, 2002). Organic lead is more evenly distributed between erythrocytes and soft tissue and less likely to accumulate in bone than inorganic lead due to its higher lipid solubility (Pattee and Pain, 2003).

In studies of mallards (*Anas platyrhyncos)* experimentally exposed to lead it was found that bone lead was much higher in laying females than non-laying females and males (Finley and Dieter, 1978) suggesting that during periods of rapid bone deposition (e.g. growth and egg laying) lead is rapidly deposited into bone (Pain, 1996).

4.2.3 Metabolism and Excretion

Lead is a cumulative toxin with a very long half-life (Lauwerys and Hoet, 2001). There are inter-specific differences in the half-life of lead in tissues (Pattee and Pain, 2003), however there is also likely to be significant individual variation. Blood, liver and kidney lead levels indicate fairly recent absorption and bone levels indicate both recent and past absorption of inorganic lead, with older animals commonly having higher bone levels (Pattee and Pain, 2003), depending on physiological state when exposed. Lead storage and mobilisation in avian bone is dependent on sex and physiological state, as bones act as a calcium store that is rapidly mobilised for egg production in female birds (Pattee and Pain, 2003).

Excretion of absorbed lead primarily occurs through the kidneys with 60 to 75 % reported to be excreted by this route in humans (Anderson et al., 1996; Lauwerys and Hoet, 2001). This is thought to be due to the shedding of tubular epithelial cells containing intra-nuclear lead as a protein complex as well as glomerular filtration (Gwaltney-Brant, 2002; Dart et al., 2004). However, the lead level in bile is much higher than lead in urine, suggesting that lead that is excreted through the bile is reabsorbed and subsequently excreted through the kidneys (Lauwerys and Hoet, 2001).

4.3 Mechanisms of Toxicity

Lead acts at the molecular level to inhibit many enzymes essential for normal biological function (Pattee and Pain, 2003). Needleman (2004) summarises some of the effects noting enzyme and structural protein disruption; interference with endogenous opiate system development; cleaving tRNA; and calcium mimicry and agonist behaviour. Lead binds sulfhydryl groups, inhibits membrane associated enzymes and can alter vitamin D metabolism (Gwaltney-Brant, 2002). As well as affecting cellular respiration at a mitochondrial level, lead has been shown to compete with calcium and

affect neuronal signalling even at picomolar concentrations (Needleman, 2004). There are many calcium dependent biological mechanisms that can potentially be affected by lead. Lead is also deposited into calcium rich structures such as bone (Gwaltney-Brant, 2002; Hontela and Lacroix, 2006). Inactivation of enzymes such as those active in haeme synthesis occurs due to the high affinity of lead for sulfhydryl groups (Gwaltney-Brant, 2002). Increased red blood cell fragility, renal tubular damage and hypertension (in humans) are reported as being due to inhibition of membrane associated proteins such as the sodium-potassium pumps (Gwaltney-Brant, 2002).

5.0 Clinical Effects on Body Systems

The effect of lead on body systems varies depending on the level and duration of exposure, inter- and intra-specific variation and stage of development. Biological systems are affected by the lowest measurable lead concentration (Pain, 1996), and probably by levels below that which we can currently detect. Increased susceptibility to predation, starvation and infection can occur due to the physiological and behavioural changes outlined below (Fisher et al., 2006). There are many studies of the effects of lead on animals under experimental conditions. While they are useful, variation may occur under natural conditions due to differences in absorption and distribution being influenced by biological and environmental factors (Pattee and Pain, 2003). Exposure to multiple toxins in their environment as well as dietary and physiological effects such as age and reproductive status may lead to different effects of lead (Pattee and Pain, 2003) in an individual or a population.

5.1 Central Nervous System (CNS)

5.1.1 Pathophysiology

Lead has direct and indirect toxic effects on neurons, astrocytes and cerebral endothelial cells (Zachary, 2007). Lead is able to cross the blood brain barrier (BBB) by substituting for calcium in a cationic transport pump; it then enters cells using voltage-sensitive cell membrane calcium channels (Zachary, 2007). Metabolic pathways and neurotransmitter systems are disrupted and calcium homeostasis is altered resulting in apoptotic cell death (Zachary, 2007). In endothelial cells this can lead to vascular leakage and eventual cerebral oedema, with laminar cortical necrosis in severe cases (Maxie and Youssef, 2007; Zachary, 2007). Lead also alters the permeability of the

BBB in developing brains by affecting the junctions between the barrier cells (Regan, 1993). Due to greater lead uptake, incomplete development of metabolic pathways and increased permeability of the blood brain barrier, young animals are at increased risk of adverse effects of lead exposure compared with adults (Pattee and Pain, 2003).

5.1.2 Developmental Changes

Studies using rats have shown that low levels of lead exposure can cause brain development alterations such as a reduction in the thickness of the cerebellar molecular layer, stunting of Purkinje cell dendrite arborisation and reduced cortical synaptogenesis and complexity, as well as hippocampal damage (Verity, 1997). Reddy, Devi and Chetty (2006) in a study of developmental lead neurotoxicity found that lead exposure alters brain acetylcholinesterase (AChE) and acetylcholine activity in young rat brains more significantly than adult rat brains.

The integrity of the blood brain barrier as well as the development of myelin and collagen can also be affected (Needleman, 2004), although this is thought to be primarily a problem of immature animals (Anderson et al., 1996). Lead has effects on child brain development (Needleman, 2004) leading to a range of neurological disorders and deficits such as learning disabilities and anti-social behaviour (Anderson et al., 1996). Regan (1993) found that lead decreased the number of synapses and neurotransmitter receptors in the developing brain of rats.

5.1.3 Cognition and Behavioural Effects in Humans

While acute encephalopathy is more likely to occur in young animals and with high doses (Yasutake and Hirayama, 2002), even extremely low lead concentrations can affect CNS function in developing nervous systems (Anderson et al., 1996). Despite disagreement in the literature as to the importance of low-level lead exposure on intelligence and learning over social factors such as parenting and nutrition in children, there is general agreement that there is a significant effect of lead on their intellectual and behavioural function (Winder, 1993; Koller et al., 2004; Koller et al., 2005). It has been shown that long term cognitive function, attention, and behaviour can be severely affected in children that have survived encephalopathies due to acute lead poisoning. Long term studies of children have shown that the effects of elevated lead levels in early childhood can persist into at least early adulthood and can result in reading disabilities, low academic achievement levels, and problems with fine motor function (Needleman,

2004). Studies looking at the relationship between lead exposure and delinquency found that pre- and postnatal lead exposure resulted in increased occurrence of aggressiveness and delinquency (Needleman, 2004). Behavioural changes due to lead toxicity in humans have been modelled using rodents (Cory-Slechta, 1988) and primates. In a study of primates, impaired social function was found to occur (Needleman, 2004).

5.1.4 Behavioural Effects in Birds

Behaviour can be a sensitive indicator of toxicity as changes can be seen at levels less than that causing mortality (Peakall, 1985; Hoffman, 2003).While there are few studies looking at the behavioural outcomes from lead induced CNS disturbance in young avian species (Scheuhammer, 1987), lead exposure has been demonstrated to affect neural function and behaviour in birds. In herring gulls (*Larus argentatus)* and common terns (*Sterna hirundo),* neurobehavioural development was affected by lead exposure and varied with dose, time of exposure and tissue level (Burger and Gochfeld, 1997, 2000, 2005). In wild and laboratory birds, low level lead was found to affect growth, locomotion, balance, food begging, feeding, thermoregulation, depth perception and recognition of individuals (Burger and Gochfeld, 2000). However, wild birds recovered quicker and reached equivalent fledging weights as controls, probably due to parental compensation (Burger and Gochfeld, 2000).When examining whether control chicks learned a given task faster that lead-affected chicks, they found that for more complex learning tasks, lead-affected chicks performed poorly (Burger and Gochfeld, 2005). Burger and Gochfeld (2000) used their work in herring gull and common tern chicks to establish an experimental protocol for examination of avian neurobehavioural development. Douglas-Stroebel et al (2005) examined time-activity budgets of mallard ducklings fed diets containing lead-contaminated sediment from the Coeur d'Alene river basin and found that bathing and swimming was affected in addition to lead affected birds experiencing poor growth, impaired balance and mobility problems. The behavioural effects of lead may lead to decreased survival of an individual animal through, for example, reduced predator avoidance and reduced ability to seek food or shade (Burger, 1995). Due to the difficulties of field-based behavioural studies, there are few studies available on the effects of lead on wild animals' behaviour (Burger, 1995).

5.2 Peripheral Nervous System (PNS)

5.2.1 Pathophysiology

Axons are targeted by lead leading to segmental demyelination and axonal degeneration, particularly in motor nerves (Dart et al., 2004; Maxie and Youssef, 2007). Lead induced demyelinating neuropathy is thought to be caused by toxicity to Schwann cells due to increased endoneurial lead (which occurs due to increases in plasma lead) (Verity, 1997). Affected Schwann cells become anoxic leading to demyelination which may progress to axonal degeneration (Anderson et al., 1996). Demyelination results in generalised weakness and symptoms such as foot drop and hand drop in humans (Anderson et al., 1996), and leg and wing paresis or paralysis in birds. Disease in peripheral nerves may precede disease in other tissues producing clinical signs in some bird species such as mallards (Hunter and Wobeser, 1980).

5.2.2 Diagnosis

In humans, PNS disease is more often diagnosed in adults than in children (Anderson et al., 1996), however sub-clinical disease may occur at any age. Feldman (1977) found that PNS disease as measured by motor conduction velocity (MCV) was present in both clinical and sub-clinical subjects. In that study, MCV along with vague clinical complaints resolved with chelation therapy (Feldman, 1977).

5.3 Haematology

Haematological effects appear to be common across most vertebrate species examined (Pattee and Pain, 2003). During haemoglobin production in red blood cells, aminolevulinic acid dehydratase (ALAD) acts to form porphobilinogen by condensing two molecules of aminolevulinic acid (Melancon, 2003). This enzyme is very sensitive to lead with inhibition following even very low exposure occurring in a wide range of species, including birds (Pattee and Pain, 2003). Haeme synthetase is also inhibited by lead, leading to reduction in ferrous iron incorporation into protopophryrin IX (Anderson et al., 1996; Pattee and Pain, 2003). These enzyme inhibitions can lead to decreased haemoglobin concentration and anaemia with both acute and chronic exposure (Pattee and Pain, 2003). Increased red blood cell fragility, decreased life-span and decreased production can occur with lead exposure and may also result in anaemia (Pattee and Pain, 2003). Abnormal accumulation of ribosome aggregates in red blood cells leads to basophilic stippling (Anderson et al., 1996). This is classically a

mammalian feature of lead poisoning however it has also been described in avian red blood cells (Campbell and Ellis, 2007).

5.4 Other Body Systems

5.4.1 Bone

With chronic exposure, lead accumulates in the mineral phase of bone. Lead becomes relatively inert when it becomes bound in the bone matrix and until the latter half of last century was considered to be permanently sequestered (Anderson et al., 1996). Lead forms stable complexes with phosphate, replacing calcium in hydroxyapatite and is deposited in bone during mineralization and released from bones during remodelling and resorption (Anderson et al., 1996; Lauwerys and Hoet, 2001). Considerable amounts of lead (greater than half of that absorbed) can be stored in bone (Lauwerys and Hoet, 2001). Pattee and Pain (2003) suggest that the toxic effects of lead may be decreased in young growing birds (compared to adults) due to increased sequestration of lead into rapidly growing bones. Bone lead is mobilised when bone turnover is increased, such as in reproduction, osteoporosis and fracture, elevating blood lead levels and potentially causing recrudescence of clinical or development of sub-clinical disease (Needleman, 1980). Trabecular bone turns over faster than cortical bone and lead is exchanged rapidly between bone surfaces exposed to blood (Lauwerys and Hoet, 2001). Studies of postmenopausal women have shown an increase in blood lead levels as their trabecular bone becomes demineralised (Needleman, 2004). In mallards experimentally exposed to lead, there was variation in the way lead was absorbed depending on sex and the season with females depositing more lead in the bones during the breeding season (Rocke and Samuel, 1991). Mobilised bone-lead may affect female birds and their offspring when they begin reproducing and bone turnover is increased for calcium deposition into egg shells. No adverse effects were identified in a study of lead in kestrel eggs (Pattee, 1984), however lead was found in the shell and yolk from lead affected chickens and the frequency of laying cycles was increased compared to those not exposed to lead (Mazliah et al., 1989).

5.4.2 Renal System

Damage to the renal proximal tubular epithelial cells may occur with severe acute lead intoxication resulting in problems with solute transfer mechanisms (Anderson et al., 1996). Sodium-potassium pumps may also be directly affected by lead

compounding the solute transfer impairment in lead affected cells (Gwaltney-Brant, 2002). Destruction of renal tubular cells and subsequent fibrosis can result (Harbison, 1998), potentially leading to renal dysfunction or failure if sufficient renal tissue has been damaged. Acute renal failure may result from very severe acute intoxication while chronic renal disease may result from more chronic exposure. Intra-nuclear inclusions are formed when non-histone nuclear proteins present in the nucleus of the renal tubular cells bind lead (Gwaltney-Brant, 2002).This may initially help mitigate the effects of the lead by taking it out of circulation, but toxic damage to the renal tubular cells results in necrosis and sloughing of cells.

5.4.3 Immune System

The susceptibility of an animal to disease may be increased by subclinical lead poisoning (Pattee and Pain, 2003). Lead can cause functional changes in the immune system (Dietert et al., 2004). One of these changes is alteration of the proportion of T-helper 2 (increased) and T-helper 1 cells (decreased), modifying the immune systems response to disease (Dietert et al., 2004). Macrophage function can also be affected (Blakely and Archer, 1981). Immune system effects vary depending on dose of lead and the life stage of the organism (Dietert et al., 2004). Lead can cause decreased production of antibodies, predisposing to infectious diseases (Parton et al., 2001). Disease caused by *Salmonella typhimurium* was increased in rats exposed to sub-clinical levels of lead (Hemphill et al., 1971). Leucocytosis has been shown to occur in mice fed moderate levels of lead for up to ten days, but was not observed with longer dose regimes (30 days) (Schlick and Friedberg, 1981). Following exposure to endotoxin, all of the lead exposed mice exhibited poor reticulo-endothelial cell responses compared to control mice (*Mus usculus)* (Schlick and Friedberg, 1981). Rocke and Samuel (1991) found that male mallards exposed to lead in the field and via intubation experienced a decline in mean circulating white cell count (WCC) when compared to controls, however females did not. The authors considered seasonal effects as the reason for this finding (Rocke and Samuel, 1991). Antibody production was found to decrease in mallards within a few days of being dosed with lead shot and persisted while blood lead levels were decreasing (Trust et al., 1990). However, in a study of the effects of a non-pathogenic immunological challenge to lead-shot exposed western bluebirds (*Sialia mexicana*), Fair and Myers (2002) did not observe immunosuppressive effects of lead except in the high dose group in which cell-mediated immunity was depressed. However they used smaller

amounts of lead shot than other studies examining immune system effects of lead (Fair and Myers, 2002).

5.4.4 Reproductive and Endocrine Systems

Lead is also known to be an endocrine disruptor and can cause reproductive and developmental abnormalities in wildlife (Hoffman, 2003). Lead exposure is reported to cause decreases in serum thyroxine levels, as well as alterations in adrenal hormones and in vitamin D metabolism, however the mechanisms are currently unknown and the clinical significance is not yet clear (Gwaltney-Brant, 2002; Dart et al., 2004). Ronis *et al* (1996) showed that lead has an effect on the hypothalamic-pituitary axis in *in utero* exposed male rats resulting in reductions in testosterone and subsequent decrease in luteinising hormone (LH) and secondary sex organ weight. Female pups in the same study experienced decreased LH and mean serum oestradiol concentrations leading to a disruption of oestrus cycling (Ronis et al., 1996).

Lead can cause changes in implantation, embryonic development and reproductive organs in mammals (Gross et al., 2003). A study in male rats showed a variety of adverse reproductive effects including impaired sperm motility (Gross et al., 2003). Lead is also associated with impaired spermatogenesis and increased miscarriages in humans (Winder, 1993; Harbison, 1998; Mackenzie and Hoar, 2002; Sikka and Naz, 2002). However conflicting studies on human male reproductive capacity are found in the literature, probably due to confounding occupational or social factors not being identified or examined (Gidlow, 2004).

6.0 Environmental Lead

"Lead is toxic wherever it is found…and it is found everywhere!"(Berney, 1996)

Natural environmental lead is rare and is emitted through volcanic eruptions and weathering of rocks (Nordic Council of Ministers, 2006). However, since the industrial revolution, anthropogenic emissions of lead have increased environmental levels significantly (Pattee and Pain, 2003). Although lead emissions are generally concentrated in industrial areas, small particles have been transported in winds to even the most remote environments including the Poles (Pattee and Pain, 2003). Despite

increasingly strict regulations in developed countries on lead usage and emissions (such as the phasing out of lead in petrol), environmental sources of lead are still a common cause of wild animal morbidity and mortality (Pattee and Pain, 2003). Animals living in urban environments or those exposed to point sources of lead emission such as near mines, factories and shot-over areas are more likely to have higher tissue lead levels (Pattee and Pain, 2003).

Wild avian species are exposed to lead through ingestion of lead shot from the environment or carcasses, fishing sinkers, lead paints, solders, household items, construction materials and environmental contamination from industry and urban areas such mines, smelters and rubbish dumps (Hoffman, 2003).

Lead contamination of water, atmosphere and industrial areas has been well studied (for examples see Ward, 1977; Steiner and Clarkson, 1985; New Zealand Commission for the Environment, 1986; New Zealand Ministry of Commerce, 1994; Nordic Council of Ministers, 2006) but for the purposes of this thesis, I will concentrate on lead contamination of soils and anthropogenic sources of lead of most significance to New Zealand wildlife.

6.1 Lead in Soil and Sediment

Sewage, industrial discharges and atmospheric lead are common sources for lead contamination of soil, can lead to high lead concentrations in sediments and have resulted in the death of many birds (Pattee and Pain, 2003). Soils and sediments such as in ocean beds become sinks for emitted lead (Pattee and Pain, 2003). Soil in localised areas can become heavily contaminated with metallic lead in the form of lead shot and fishing sinkers from hunting, fishing and target shooting (Pattee and Pain, 2003). This form of lead has been directly responsible for the death of a large number of water birds and upland game birds, and, indirectly, raptor species who feed on these prey (Pattee and Pain, 2003). The bioavailability of lead in soil is dependent mostly on soil pH, as well as organic matter content, clay content, cation exchange capacity and aging; the amount of total lead in the soil is generally greater than the bioavailable lead (Beyer and Fries, 2003; Booth et al., 2003). Soil ingestion may occur purposefully or accidentally with preening and feeding (Beyer et al., 1994; Beyer and Fries, 2003). Ground feeding birds may ingest soil or sediment with their food (Beyer et al., 1994). Intake of soil has been determined in some agriculturally important grazing species in New Zealand, specifically sheep and dairy cattle, however wildlife species are considerably more

difficult to assess (Beyer et al., 1994). Soil ingestion may be a source of toxin exposure in wildlife (Beyer et al., 1994). Lead toxicity in birds ingesting sediments has been studied by various authors in Europe and the United States (e.g. Chupp and Dalke, 1964; Hoffman et al., 2000a; Hoffman et al., 2000b; Sileo et al., 2001) and summarised in Beyer and Fries (2003). In studies of waterfowl feeding in the Coeur d'Alene River Basin, Idaho, sediment ingestion rather than lead shot or sinkers was identified as the cause of lead intoxication in hundreds of birds with lead poisoning (Chupp and Dalke, 1964; Sileo et al., 2001; Beyer and Fries, 2003). Lead contaminated river sediment was also fed to Canada goose (*Branta canadensis)* goslings and mallard ducklings over six weeks and found to have severe physiological effects including decreased growth and mortality (Hoffman et al., 2000a; Hoffman et al., 2000b).

6.1.1 Lead and Earthworms

Earthworms may act as bioaccumulators of metals (Reinecke et al., 2000; Booth et al., 2003; Darling and Thomas, 2005), therefore there is potential for birds, small mammals, reptiles and amphibians that feed on earthworms to obtain toxic levels of lead from this food source (Darling and Thomas, 2005). Elevated lead in tissue of wild shrews (*Myosorex varius)* was found in urban areas of South Africa and suspected to be due to consumption of earthworms containing lead (Reinecke et al., 2000). Shrews subsequently fed lead contaminated earthworms were found to accumulate higher liver lead concentrations than the controls (Reinecke et al., 2000). Different species of earthworms may accumulate different amounts of lead independent of the concentration of lead in the soil (Pattee and Pain, 2003). *Lumbricus terrestris* earthworms were found to significantly bioaccumulate both lead acetate and lead carbonate under experimental conditions, although lead acetate levels were much higher than lead carbonate, probably due to increased solubility of this compound (Darling and Thomas, 2005). Reinecke et al (2000) suggests that analysing the lead content of the actual worms may be a better indicator of the risk of lead in soil as they contain mostly bioavailable lead.

Birds may also indirectly ingest lead-contaminated soil along with invertebrate prey, either as contaminated soil on the cuticle or in the digestive tract of the prey species. Beyer and Fries (2003) suggest estimating soil lead exposure by analysing prey such as earthworms (20-30% dry weight is soil) but comment on the limitations of this method as some birds "clean" their worms prior to ingestion, thus reducing their exposure.

6.1.2 Lead Contaminated Soil and Plants

Ingestion of plants from contaminated soils may also result in lead exposure. Analysing plant material for lead is an inaccurate measure of exposure as the quantity of soil ingested will vary greatly with feeding habit. For example, some species clip the vegetation and others pull up clumps of vegetation with roots and soil (Beyer and Fries, 2003). Vegetation sampled from firing ranges was found to have accumulated lead from the soil in addition to being coated in lead containing dust (Bennett et al., 2007). This suggests that point sources such as firing ranges provide multiple risks of exposure through plant and soil ingestion as well as lead pellet ingestion. Intra- as well as inter-specific differences in sediment ingestion have been found in waterfowl (Beyer and Fries, 2003).

6.2 Lead Ammunition

An estimated 20,000 tonnes of lead in the form of ammunition is shot into the North American environment every year (Thomas, 1997) and it takes between 100-300 years for lead shot to degrade depending on local soil conditions (De Francisco et al., 2003). Lead ammunition in the soil slowly breaks down into lead compounds such as lead carbonate and lead acetate (Booth et al., 2003; Darling and Thomas, 2005). Lead shot has been responsible for the mortality of millions of birds around the world each year (Pattee and Pain, 2003). Waterfowl ingest lead shot while feeding, probably as grit or food and the lead shot remains in the gizzard and is used along with pieces of grit to grind food (Pain, 1996). This action in combination with the acidic conditions of the proventriculus facilitates the breakdown of the lead and absorption into the bloodstream (Pain, 1996). Waterbirds feeding in wetlands, birds feeding in clay target and firearm ranges, upland game birds and carnivorous birds are at most risk of exposure (Pattee and Pain, 2003). Lead shot ingestion usually occurs on feeding grounds, however in a study of lead exposure in threatened spectacled eiders (*Somateria fischeri),* the authors found that breeding animals and ducklings had radiographic evidence of lead shot ingestion, and/or elevated blood lead concentrations (Franson et al., 1998).

Mortality and morbidity of birds that feed on lead-poisoned prey or prey carrying lead shot in their tissues has been reported in a wide range of raptors around the world and is a cause of decline in many endangered raptor species including the California condor (Fry, 2003; Pattee and Pain, 2003). Increased numbers of lead-affected eagles have been found in areas of waterfowl hunting, and in one study, nearly

40 % of sick bald eagles were found to have clinical or sub-clinical (elevated blood or tissue levels) lead poisoning (Fisher et al., 2006). Lead fragments are left in the bullet tract and surrounding tissue when animals are shot, exposing scavenging birds and other animals when they feed on shot animals that are not recovered by hunters (Fisher et al., 2006).

Lead shot for waterfowl hunting has been prohibited since 1991 in the United States. (USFWS, 1995; Franson and Smith, 1999). Kramer and Redig (1997) in a review of lead poisoning in eagles over 16 years did not find a significant reduction in rates of lead exposure before and after the ban, possibly due to lead still being used in non-waterfowl hunting (Clark and Scheuhammer, 2003) or persistence of lead contaminants in the environment. However a reduction in mean blood lead concentrations in the study population was seen (Kramer and Redig, 1997). A Canadian study looking at the bone lead concentration of hatch-year mallards, American black ducks (*Anas rubripes)*, ring-necked ducks (*Aythya collaris)* and American woodcocks (*Scolopax minor)* found that the waterfowl species had up to 70% reduction in their bone lead concentration following lead-shot restrictions whereas the woodcock, an upland game species, had no reduction (Stevenson et al., 2005). The risk of poisoning from lead ammunition has not been eliminated as ammunition containing lead (including shot) is still used in non-waterfowl hunting, some hunters still illegally use lead shot and millions of birds still continue to be poisoned from lead present in the environment prior to the ban (Hoffman et al., 2002; Stevenson et al., 2005).

Despite lead shot for waterfowl hunting being banned or restricted in many countries for over ten years, New Zealand only recently brought in restrictions at the beginning of the 2005 hunting season. Use of lead shot is now prohibited within 200 metres of waterways and for hunting waterfowl in most locations; however there is no restriction on lead ammunition for hunting upland game or mammals, or if using smaller gauge shotguns (less than 12 calibre) (Fish and Game New Zealand, 2007).

6.3 Other Anthropogenic Sources of Lead

Other less common anthropogenic sources of environmental lead include fishing weights, lead-based paint, lead emissions into the air, lead-containing construction materials and lead in items disposed of to landfill. Although there are fewer areas of high concentration of fishing weights, these may cause problems in swans and other waterfowl following ingestion. Most lead fishing weights were banned in the United

Kingdom in 1986 following concern regarding the mortality of mute swans (*Cygnus olor*) (Pattee and Pain, 2003).

The bright colours and weather resistant properties of lead compounds have resulted in their extensive use in paints and other coatings (Dart et al., 2004). In New Zealand, lead in paint was legally restricted by the Toxic Substances Regulation Act (1983) however older buildings with leaded paint are still considered a significant source. Old buildings with flaking lead paint may cause local lead contamination of soils (Pattee and Pain, 2003) as well as large paint flakes on the surface. Laysan albatross chicks suffered morbidity and mortality that was linked to ingestion of lead paint flakes from old buildings on Midway Atoll, Hawaii (Sileo and Fefer, 1987). Although lead content has been reduced in more modern paints, they are not all lead free (Summers et al., 1995). While the residential use of lead paint is now banned in many countries, outdoor (such as marine) and agricultural uses are still common (Gwaltney-Brant, 2002).

Point-source emissions from heavily industrialised areas may be a significant exposure route for some birds. Several thousand nectar-feeding birds died at an industrial port in Australia following consumption and inhalation of lead carbonate dust that had settled on foliage and flowers, probably concentrating in nectar (DEC, 2007).

7.0 Assessment of Lead Exposure and Toxicity

Given the range of clinical and sub-clinical effects of lead exposure, it is not surprising that there is controversy regarding an exact definition of tissue lead concentrations consistent with lead poisoning. To make a diagnosis of lead poisoning as a cause of mortality, toxic tissue levels supported by clinical and/or pathological findings should be present (Franson, 1996; Pain, 1996; Hoffman et al., 2002). Antemortem diagnosis of lead poisoning or exposure in wildlife requires a thorough physical examination (including neurological exam) and ancillary tests. A thorough history is also useful but may not always be available.

Testing can be carried out to directly detect lead in biological samples and therefore measure exposure. Commonly used assessments include direct assessment of lead in blood and tissues and indirectly by testing to detect biological effects of lead such as its effects on packed cell volume (PCV) and delta-aminolevulinic acid (ALAD).

7.1 Antemortem Tests for Lead Exposure

7.1.1Blood Lead Concentrations

Assessing the concentration of lead in blood is the primary test used to diagnose lead exposure and is also useful in monitoring treatment (Dart et al., 2004). It is most useful as an indicator of recent exposure and of soft tissue lead concentration (when in steady state with blood lead concentration), particularly in experimental studies (Graziano, 1994; Pain, 1996; Lauwerys and Hoet, 2001). Historically, testing blood lead has been impractical for field situations and for testing small animals due to expense, the large volumes of blood required and considerable inter-laboratory variation (Pain, 1989). Fortunately recent advances in techniques for measuring blood lead have occurred enabling lower levels of detection on smaller volumes of blood, making it the most widely used and useful test for lead detection (Graziano, 1994). Portable battery powered analysers can be purchased for in-field blood lead testing on 50 µL of whole blood enabling patient-side testing. Blood lead is not however, representative of total body lead burden (Pain, 1996). When exposure to lead is ceased, the blood lead values may decrease progressively, but over a prolonged period of time, and may not decrease below the level considered normal for "background exposure" due to release of lead from stores in soft tissue and bone (Pain, 1996).

The United States Center for Disease Control (CDC) and World Health Organisation (WHO) action levels for blood lead concentration in humans is 0.1 mg/L for children less than 16 years old and 0.25 mg/L for adults (WHO, 1995). However, the Agency for Toxic Substances and Disease Registry in the United States has not established minimum risk levels for lead due to a inability to identify a clear threshold for the more subtle effects of lead (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). In New Zealand, the minimum action level is 0.15 mg/L for all age groups (Ministry of Health, 1998). In human medicine, blood lead levels greater than 0.1 mg/L are used as an indicator of potential toxicity and levels above 0.65 mg/L are treated as an emergency (Anonymous, 2005). In parrots, it has been suggested that blood lead levels greater than 0.2 mg/L are suggestive of acute lead toxicity and if accompanied by clinical signs, levels greater than 0.4-0.6 mg/L are diagnostic for lead toxicity (Dumonceaux and Harrison, 1994). Needleman (2004) reports that in humans, clinical symptoms are rarely seen below 0.6 mg/L, however levels greater than 0.1 mg/L are considered elevated.

7.1.2 Haematology

Haematology is not always useful in the early diagnosis of lead exposure in an individual, however, slight reductions in PCV and/or haemoglobin may be seen when looking a trends in a population (Lauwerys and Hoet, 2001). Anaemia can be an early indicator of chronic exposure (Harbison, 1998). In humans, normochromic, normocytic anaemia with stippling may be seen with severe intoxication. Approximately half of lead affected cats (*Felis catus)* and dogs (*Canis familiaris)* will have nucleated erythrocytes and a quarter will have basophilic stippling (Parton et al., 2001). Basophilic stippling associated with lead exposure is rarely seen in avian red cells (Campbell and Ellis, 2007) and anaemia may have many causes, thus the use of haematology as an indicator of lead toxicity in birds is limited.

7.1.3 Other Tests

Red Blood Cell Delta-aminolevulinic Acid Dehydratase (ALAD)

ALAD is active in the synthesis of haemoglobin and is very susceptible to inhibition by lead (Lauwerys and Hoet, 2001; Melancon, 2003). Hoffman et al (2002) feel that ALAD suppression is directly correlated with blood lead level and this view is supported by Yasutake and Hirayama (2002). Conversely, in Harbison's (1998) opinion, ALAD measurement has limited usefulness as blood levels do not correlate closely with lead exposure. This may be because it is unable to differentiate between moderate and severe toxicity because significant inhibition of ALAD occurs at low levels of blood lead concentration (< 0.30 mg/L) (Graziano, 1994). ALAD must also be measured within 24 hours as it is chemically unstable (Graziano, 1994; Harbison, 1998). This would make it less useful for situations where distance to the laboratory is increased.

Radiology

Radiology can be used to detect radio-opaque items in the gastrointestinal tract. In some cases multiple bands of increased density (sclerosis) due to persistence of mineralised cartilage in the metaphyseal margins (lead lines), may be seen on radiographs (Maxie and Youssef, 2007).

Isotope Typing

As lead occurs in several different isotopes (^{204}Pb, ^{206} Pb, ^{207}Pb and ^{208}Pb) that occur in different quantities in different locations, detection of the isotope types can

assist in identifying environmental sources of lead (Graziano, 1994). The source of lead affecting red kites (*Milvus milvus)* in England was identified using this method on tissue of lead affected birds and potential environmental lead sources (Pain et al., 2007). In a study by Svanberg et al (2006) lead isotope typing identified the source of lead for dead or moribund marbled teal (*Marmaronetta angustirostris)* and white-headed ducks (*Oxyura leucocephala)* to be lead shot. In addition the authors found that marbled teal ducklings also had bone lead consistent with exposure to lead shot suggesting that the lead had been obtained via the hens which had ingested lead shot (Svanberg et al., 2006).

7.2 Post-mortem Testing of Lead Exposure

It is important to distinguish lead poisoning as a *cause* of mortality from lead poisoning as a disease (Franson, 1996). Gross pathological and microscopic lesions vary between peracute, acute, subacute and chronic exposure, quantity of lead and between species. Lesions may not be observed with peracute poisoning, however items containing lead may be found in the gastrointestinal tract (Zachary, 2007). Lesions are generally absent or non-specific and additional toxicology testing of tissues is usually required to diagnose lead poisoning or exposure (Beyer et al., 1998; Maxie and Youssef, 2007).

7.2.1 Gross Lesions

Lesions are usually not observed in the CNS, but if present in mammals may include haemorrhage, swelling and resultant flattening of gyri, and vascular congestion of the meninges and cerebrum (Zachary, 2007). In more chronic disease, malacia in the cerebral cortex and laminar necrosis may be seen, progressing to loss of white matter (Zachary, 2007). Oesophageal and proventricular impaction, enlarged gall bladder and green-stained koilin are often seen in waterfowl poisoned by lead (Franson and Smith, 1999).

7.2.2 Histopathology

CNS lesions visible at histopathological examination of tissues may include variable degrees of congestion, swelling of astrocytes, status spongiosis, endothelial hypertrophy and ischemic neuronal cell change in acute cases of lead poisoning in cattle (Zachary, 2007). In caged birds affected by lead, neuronal degeneration may be seen in

the cerebral cortex with shrunken basophilic angular neurones, enlarged perivascular spaces and leptomeningeal oedema (Jones and Orosz, 1996). However, despite clinical evidence of neurological disease, microscopic evidence of cerebral oedema may not be appreciated (Maxie and Youssef, 2007). Mural hyalinisation, necrosis and thrombosis may result from vascular lesions and Purkinje cell necrosis can be observed throughout the brain. Destruction of myelin in the cerebrocortical white matter and the PNS can occur, the latter leading to a peripheral neuropathy (Zachary, 2007). In waterfowl experimentally dosed with lead, areas of cerebellar microhaemorrhages were seen with swollen endothelial cells and occasionally hyaline thrombi (Hunter and Wobeser, 1980).

While peripheral nervous system changes are species dependent, segmental demyelination with variable axon degeneration are usually common features (Verity, 1997). Different stages of demyelination and remyelination may be present as lead neuropathy usually develops following prolonged exposure (Feldman, 1977). Hunter and Wobeser (1980) found swelling and fragmentation of myelin nerve sheaths occurring in the vagus, brachial and sciatic nerves of lead poisoned mallards.

Lead is a nephrotoxin and can cause toxic damage to the epithelial cell membranes of the proximal convoluted tubules (Newman et al., 2007). Occasionally mild degenerative changes are observed in the liver and kidney and acid-fast intra-nuclear lead inclusions may be observed in the renal tubular cells, and occasionally in hepatocytes in sub-acute to chronic cases (Maxie and Youssef, 2007). Lead inclusions may also be observed in the nucleus of osteoclasts, particularly in cases of lead induced sclerosis of the metaphyseal margins (Thompson, 2007). Muscle fibre degeneration and disintegration with no inflammatory response has been observed in experimentally dosed mallards (Clemens et al., 1975).

7.2.3 Tissue Analysis

Although potentially useful for antemortem diagnosis via surgical collection of tissues, tissue lead levels are usually reserved for post-mortem diagnosis. Liver and kidney are the most commonly tested organs for tissue lead levels although lead can be stored in a range of tissues (Summers et al., 1995). In a study of lead in bald and golden eagles (*Aquila chyrsaetos)*, Wayland *et al* (1999) found similar lead results in levels in kidney and liver samples at low levels of exposure, but at higher levels of exposure, the lead in liver tissue was in most cases higher than that in kidneys. This is in contrast with

several other studies that found renal lead levels to be higher than liver (Wayland et al., 1999). The authors conclude that it is advisable to test both liver and renal tissue when lead poisoning is suspected. Over time, considerable lead can be stored in bone making bone lead a useful indicator of chronic lead exposure (Pain, 1996). Clinical effects of lead exposure in individuals can easily be related to lead concentration in blood and soft tissues, but not bone (Pain, 1996). However on a population level bone lead analysis can enable determination of geographical patterns of lead exposure and can be correlated with liver lead level (Pain, 1996).

Interpretation of the clinical significance of tissue lead levels without knowledge of history, clinical presentation or pathological findings is difficult due to differences in acute and chronic exposure and factors such as age, sex, reproductive status and species (Pain, 1996). Tissue levels of lead do not always correlate with clinical disease or pathological changes and there is considerable inter- and intra-specific variation (Summers et al., 1995). Birds may die following acute exposures with high blood and liver lead levels and low bone levels or the reverse with chronic exposure (Pain, 1996). Following reviews of the literature, tissue lead values to indicate sub-clinical, clinical and toxic exposure in anseriformes, falconiformes columbiformes and galliformes, have been suggested by Pain (1996) and Franson (1996) (Table 1.3). In other species, tissue lead can be more difficult to interpret. Franson (1996) suggests making an informed diagnosis based on clinical and pathological findings combined with tissue residues. If only tissue levels are available, a conclusive diagnosis of mortality due to lead poisoning cannot be made, but it can be determined if the birds were exposed to lead and the potential consequences of that exposure can be predicted (Franson, 1996). Due to significant variation in susceptibility to lead between species, comparisons should be made between phylogenetically related groups if this data is available (Franson, 1996).

Table 1.3. Interpretation of Tissue Lead Concentrations in Various Wild Avian Species. *Background exposure* is the exposure to lead from the environment far from point sources such as wetlands containing shot and smelters. *Subclinical exposure* – physiological effects only. *Clinical (toxic) exposure* – clinical signs are visible. *Death* – death seen at these levels in the field, captivity or laboratory. NB: Animals may be more or less severely affected from exposure than these ranges predict.

Species/order	*Blood mg/L*	*Liver mg/kg (WW)*	*Kidney mg/kg (WW)*	*Bone mg/kg (DW[a])*	*Reference*
Waterfowl					
"background"	< 0.2	< 2	-	<10	Pain 1996
Subclinical poisoning	0.2 < 0.5	2 < 6	-	10-20	Pain 1996
Clinical poisoning	0.5-1.0	6-15	-	10-20	Pain 1996
Severe toxicity	> 1.0	>15	-	>20	Pain 1996
Falconiformes					
Subclinical	0.2-1.5	2-4	2-5	-	Franson 1996
Toxic	>1.0	>3.0	>3.0	-	Franson 1996
Death	> 5.00	>5.0	>5.0	-	Franson 1996
Columbiformes					
Subclinical	0.2-2.5	2-6	2-20	-	Franson 1996
Toxic	>2	>6	>15	-	Franson 1996
Death	>10	>20	>40	-	Franson 1996
Galliformes					
Subclinical	0.2-3	2-6	2-20	-	Franson 1996
Toxic	>5	>6	>15	-	Franson 1996
Death	>10	>15	>50	-	Franson 1996

Table 1.3 Continued

Species/order	*Blood mg/L*	*Liver mg/kg (WW)*	*Kidney mg/kg (WW)*	*Bone mg/kg (DW[a])*	*Reference*
Levels reported to cause morbidity or mortality in the following species					
• Laysan Albatross	0.13 –4.8	6-110	44	-	Sileo & Fefer 1987
• Passeriformes	-	20-40	-	-	Franson 1996
• Charadriiformes	-	8-31	-	-	Franson 1996
• Gruiformes	-	30	-	-	Franson 1996
• Ciconiformes	-	40-250	-	-	Franson 1996
• Gaviformes	-	20-50	-	-	Franson 1996
• Strigiformes	-	15-67	-	-	Franson 1996
• Procellariiformes	-	2-36	-	-	Franson 1996

Table modified from Pain (1996) and Franson (1996)

[a] Dry weight (DW) values are more reliable and consistent than wet weight (WW). 1ppm WW ~3-4ppm DW

8.0 Lead in New Zealand

Lead is ubiquitous throughout the New Zealand (NZ) environment due to its presence in many products in general use by society such as shot, petrol, paints and batteries. In 1986, a report by the NZ Ministry for the Environment estimated that 5000 tonnes of lead was imported into NZ annually, mainly for use in paint, solder for food and beverage cans and petrol (New Zealand Commission for the Environment, 1986). In a review of heavy metals in the NZ atmosphere, Steiner and Clarkson (1985) reported that lead in rain from the Christchurch area and lead levels in central Auckland were greater than the US Environmental Protection Agency (EPA) standard. In 1977, Ward examined the blood lead concentrations of domestic animals in New Zealand and found that urban dogs had elevated blood lead compared with rural dogs (Ward et al., 1977). The author also found that blood lead levels of sheep grazing on feed from roadsides were elevated compared with sheep grazing away from roadsides (Ward, 1977). It is likely that petrol was the source of lead in this study, however this source of lead in the NZ environment has subsequently been removed with the phase out of leaded petrol (New Zealand Ministry of Commerce, 1994). In studies of heavy metals in sediments, higher than background levels of lead were found in Porirua harbour (north of Wellington) (Glasby et al., 1990), Wellington harbour (Stoffers et al., 1986), Manukau harbour (west of Auckland) (Aggett and Simpson, 1986; Glasby et al., 1988) and Waitemata harbour (East of Auckland) (Glasby et al., 1988).

There are few published studies of heavy metal concentrations in NZ animals, the majority being restricted to studies of marine and freshwater invertebrates (e.g. Neilsen and Nathan, 1975; Zauke et al., 1992; Blakely and Harding, 2005). The first published study of lead poisoning in New Zealand waterfowl was by Wisely and Miers (1956) who examined black swans that had died at Woodend Lagoon near Christchurch. The lead was ingested in the form of lead shot which was found in the gastrointestinal tract (Wisely and Miers, 1956). Thompson and Dowding (1999) looked at heavy metal concentrations in South Island pied oyster catchers *(Haematopus ostralegus finschi*) overwintering in Mangere Inlet, Manakau harbour (polluted site), and South Kaipara harbour (non-polluted site). Mean blood lead concentrations of birds in Mangere Inlet were found to be 0.14 mg/L compared with 0.06 mg/L in those at South Kaipara Harbour (Thompson and Dowding,

1999). Juvenile birds in Mangere Inlet were found to have significantly higher blood lead levels (mean = 0.18 mg/L) than adult birds (mean = 0.11 mg/L) (Thompson and Dowding, 1999). These results are consistent with findings by Glasby et al (1988) who found lead concentrations in sediments from Manakau Harbour to be elevated. Lock et al (1992) in a study of heavy metals in sea birds from the New Zealand region found that lead concentrations in tissues were generally low or below the limit of detection of the test, particularly for pelagic birds. However, the gulls (*Larus spp.)* and cape pigeons (*Daption capense)* examined were found to have higher mean bone lead concentrations, consistent with feeding in shore and having access to contaminated food sources on land (Lock et al., 1992). A study by Turner *et al* (1978) looking at levels of pollutants including lead in selected estuarine birds throughout New Zealand, found elevated bone lead levels in individual birds from all locations studied. The authors concluded that the high levels may have been due to lead shot ingestion (Turner et al., 1978). The birds were collected by shooting and the authors do not state tissue sample collection procedures used to avoid contamination of the samples or if gastrointestinal tracts were examined for the presence of lead shot.

With evidence of environmental lead due to prior or current land use throughout New Zealand it is possible that NZ avifauna, including those that are endangered and endemic, may be exposed to lead in the environment.

9.0 Objectives and Structure of Thesis

The research presented in this thesis aimed to examine the effect of lead on selected wild bird populations in New Zealand. Kea (*Nestor notabilis)* were selected following examination of necropsy records of kea showing evidence of elevated tissue lead levels (unpublished, IVABS Post Mortem Database). Inquisitive species such as the kea may ingest lead while chewing on products containing lead and suffer subsequent mortality or morbidity. Elevated blood lead concentrations were found in kea foraging at the Halpine Creek dump, Arthurs Pass (Jarrett, 1998). Takahe *(Porphyrio hochstetteri)* were selected after two takahe from Kapiti Island presented for veterinary attention and were found to have elevated blood lead concentrations and metal densities in their gizzards. Scavenging and carnivorous birds such as Australasian harriers (*Circus approximans)* may ingest lead

when feeding on lead affected animals, or animals containing lead shot in their tissues. These birds are commonly presented to the New Zealand Wildlife Health Centre, Massey University and are often found to have elevated blood lead concentrations (unpublished).

The objectives of this study were to:

1. survey lead levels occurring in wild kea visiting Aoraki/Mt Cook village and surrounding areas; review pathological findings from kea in database archives; analyse tissue lead concentrations in archived tissue samples, and explore the potential sources of lead in the kea's environment;

2. survey lead levels occurring in free-ranging takahe occurring on Mana, Tiritiri Matangi and Kapiti Islands; review clinical and pathological findings from takahe in database archives; analyse tissue lead concentrations in archived tissues and explore the potential sources of lead in the takahe's environments; and

3. investigate the pathogenesis and prognosis of a syndrome of peripheral neuropathy in harriers presenting at the New Zealand Wildlife Health Centre and establish if it is associated with lead exposure.

4. use the findings from objective one and two to assist the New Zealand Department of Conservation develop management plans with regard to kea and takahe and lead.

The thesis is structured in five chapters. Chapter one is this literature review. Chapter two documents an investigation into lead levels in kea. Chapter three documents an investigation into blood and tissue lead levels in takahe from Mana, Kapiti and Tiritiri Matangi Islands and tissue lead levels in birds from the Murchison Mountains. Chapter four documents an investigation into peripheral neuropathy in Australasian Harriers and chapter five presents a general discussion, conclusions and recommendations. Additional methodology and other information are presented as appendices. Formatting varies slightly between chapters due to the nature of the investigations.

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"If we were to judge of the interest excited by any medical subject by the number of writings to which it has given birth, we could not but regard the poisoning by lead as the most important to be known of all those that have been treated of, up the present time" (Orfila 1817)

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Chapter 2

Evidence of Lead Exposure in a Free-ranging Population of Kea (*Nestor notabilis*)

"One kea took particular interest in the building materials and began stealing nails. .. Weeks later ….the purloined nails were discovered. They had been neatly laid in the gutters of an outbuilding's corrugated iron roof, sorted according to size."
(Temple, 1996:7)

"And the keas killed themselves, choked by ingested plastic, drowned in water tanks, poisoned from chewing lead-head nails….Life was hard and dangerous in the fast lane."
(Temple, 1996:70)

Abstract

Kea (*Nestor notabilis)* are high country parrots endemic to New Zealand. They are considered threatened following a century of indiscriminate bounty hunting. While hunting is now illegal, kea experience ongoing threats from introduced mammal species and conflict with humans living in or visiting the alpine region of New Zealand. The foraging behaviour and inquisitive nature of kea has lead to incidences of foreign substance ingestion including lead. Between April 2006 and November 2007, 38 kea visiting the Aoraki/Mt Cook Village and National Park were captured and blood sampled for blood lead analysis. All birds sampled had detectable blood lead with concentrations ranging from 0.028 mg/L to 3.43 mg/L (mean = 0.428 mg/L ± 0.581). A retrospective analysis of wild kea pathology and archived samples was also carried out. Necropsy reports were reviewed and formalin-fixed liver and kidney was analysed for lead content. Seven out of 15 birds were found to have died with elevated tissue lead. General pathological findings were not consistent between birds; however 100% of birds with elevated lead exhibited Ziehl-Neelson positive intra-nuclear inclusion bodies in the renal tubular epithelial cells. This study found that lead exposure may be an important contributing factor in kea mortality. As a result of these findings, lead abatement in areas frequented by kea is being considered.

Key Words

Kea; *Nestor notabilis*; plumbism, blood lead; Pb; toxicity; free-ranging; parrot

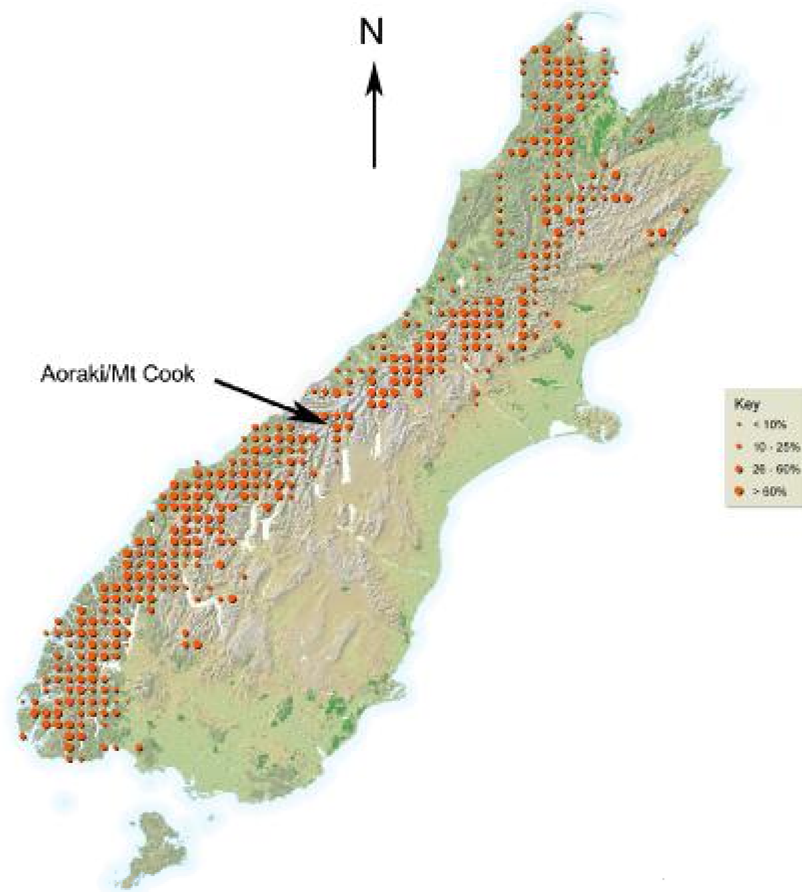
Introduction

The kea (*Nestor notabilis*) is a high country parrot living in high altitude forest and alpine basins ranging between 600 and 3000m above sea level, although it may be found occasionally descending to sea level and reaching higher elevations in summer (Forshaw, 2006). It is endemic to the high country of the South Island of New Zealand (Figure 2.1), however the fossil record indicates it was once found in the North Island (Holdaway and Worthy, 1993).

A dramatic population decline has occurred in post-European times due to a perceived threat to agriculture and following the introduction of a bounty in 1890 (Peat, 1995). It is thought that at least 150,000 kea were killed prior to 1970 when the scheme was withdrawn (Peat, 1995). Full protection of the kea was achieved in 1986 however there is no indication that the population has recovered since then (Peat, 1995). The precise size of the current population is unknown, although it is estimated to be approximately 1000-5000 birds (Pullar, 1996). Kea are listed as vulnerable by the World Conservation Union (IUCN) red list (Bird Life International, 2006), and are listed as Nationally Endangered under the New Zealand Threat Classification List (Hitchmough et al., 2007).

Potential threats to kea include: predation at nests by introduced mammals such as brush-tailed possums (*Trichosurus vulpecula*) and mustelids (*mustela spp*); competition for nest sites; degradation of habitat through fire; overgrazing by domestic stock and browsing by feral mammals; perception of kea as a pest and subsequent illegal poisoning and shooting; illegal capture and smuggling of live kea for the pet trade; misadventure (e.g. road/rail trauma, open rubbish bins), and consumption of rubbish, construction materials and handouts (Peat, 1995; Pullar, 1996; Elliott and Kemp, 2004; Bird Life International, 2006). In 1995, 27 kea killed by gunshot were found at a high country rubbish dump showing that despite protection, kea continue to be at risk of being persecuted (Peat, 1995). Until recently, conflicts with kea were confined to mainly farmers; however with increasing high-country tourism, conflicts with other groups such as local businesses and tourists are becoming more frequent (Grant, 1993; Peat, 1995; personal observation).

Figure 2.1: Distribution of kea in New Zealand showing location of study site (modified from Robertson *et al* (2007).



The bill and tongue of the kea are well suited for probing, tearing, digging and manipulation of small objects (Diamond and Bond, 1999). Kea are omnivorous, feeding primarily on vegetation and insect larvae, although they are opportunistic and will sample any potential food source (Temple, 1996). This foraging behaviour and their particularly inquisitive nature has lead to incidences of foreign substance ingestion as kea sample handouts from tourists, rubbish dumps, buildings, boardwalks, cars, farms and fishing boats (Peat, 1995; Temple, 1996). Reported causes of death in kea include lead toxicity, cyanide poisoning, rubber ingestion, methylxanthine toxicity (Gartrell and Reid, 2007), and insulation ingestion following baiting with fibreglass insulation pads (Peat, 1995). Kea that

are unwell following ingestion of rubbish items may be more at risk for road or rail deaths (Peat, 1995).

Lead is one of the most toxic metals known due to its wide ranging effects on multiple body systems, affecting many processes that are necessary for normal function (Pattee and Pain, 2003). The renal, gastrointestinal, reproductive, central nervous and peripheral nervous systems and the biosynthesis of haeme are all adversely affected by lead (Verity, 1997).

Lead toxicosis has been well described in captive parrots (Dumonceaux and Harrison, 1994) and wild waterfowl and raptors, particularly in North America and Europe (e.g. Pain, 1991; Locke and Thomas, 1996). Acute lead poisoning commonly occurs in companion parrots subsequent to chewing objects containing lead such as some bird toys, jewellery, lead paint and putty on window frames and lead-light glass (Dumonceaux and Harrison, 1994). Regurgitation/vomiting, seizures or other neurological disease, and occasionally anorexia, diarrhoea and polyuria may be seen (Dumonceaux and Harrison, 1994). Wild birds (especially waterfowl) more typically present with chronic lead poisoning. Birds are often presented dead although they may be found emaciated with paresis/paralysis of the wings and/or legs and oesophageal impaction (Locke and Thomas, 1996). Death can result from secondary infection, starvation, predation or misadventure.

The only previous study of blood lead concentrations in wild kea in which the author sampled 11 birds foraging at a rubbish dump, found that young birds were much more likely to have elevated lead levels (Jarrett, 1998). This study may not have been representative of other kea populations as only 11 birds were sampled and it was biased towards birds foraging in the rubbish dump. Lead poisoning has been recorded in several captive kea in New Zealand (unpublished data) and overseas (Zook et al., 1972). Jackson (1969) reported on causes of death of kea collected over 10 years in the Arthur's pass region of the southern high country and while in some birds clinical and pathological findings were consistent with lead toxicosis, tissue lead levels were not evaluated in this study. To the best of the authors' knowledge, this is the first report in the peer-reviewed literature of lead toxicity in free-ranging parrots.

Our study aimed to characterize the lead concentrations of free-living kea in New Zealand by:

1. surveying the blood lead levels in wild kea visiting Aoraki/Mount Cook Village and national park;
2. reviewing pathological findings from kea in database archives (the New Zealand wildlife post-mortems database 'Huia' and the Massey University post-mortems database); and
3. analysing lead concentrations in archived kea tissue samples.

Materials and Methods

Study Location

Aoraki/ Mt Cook National Park (43º73'66"S, 170º10'40''E) is located in the South Island of New Zealand (Figure 2.1). Huts for mountaineers and hikers are present throughout the park. Aoraki/Mt Cook Village is situated in a glacial valley on the east side of Aoraki/ Mt Cook, the highest peak in the Southern Alps. The village is 760m above sea level. The resident population of the village is approximately 300 people and is conservation and service based. High numbers of tourists visit the area, particularly in summer as accessibility in winter is difficult due to adverse weather conditions.

Sample Collection and Analysis

A total of 38 kea were sampled between April 2006 and November 2007 (excluding winter – June to August). Thirteen juvenile male kea, 18 fledgling kea (16 male and two female), three sub-adult kea (two male and one female) two adult male kea and two nestling kea were captured in Aoraki/ Mt Cook Village and national park.

Immediately following capture the birds were blood sampled from the left or right ulna and/or medial metatarsal vein. For the birds sampled in 2006, two smears for microscopy were made with fresh blood. Blood was placed into 0.4 ml lithium heparin microtainers (Becton Dickenson Vacutainer Systems, Preanalytical Solutions, Franklin Lakes, NJ 07417). A clinical examination was carried out on each of the birds sampled in 2006. A combination of bill size and coloration, eye, cere and crown feather coloration, moult stage and weight (when available) was used to identify the age-class and sex of the

unbanded kea. Banding records were checked to confirm age and sex of banded kea. The condition of each bird was estimated using the prominence of the keel and the pectoral muscles, and the presence of subcutaneous fat deposits (Doneley et al., 2006) and scored out of five with one being emaciated and five being grossly obese.

All unbanded birds were banded before release. Coloured bands were placed on the right tarsometatarsus to enable subsequent visual identification without capture. Steel bands with identification numbers were placed on the left tarsometatarsus.

Blood was analysed for lead content using a portable lead analyser (LeadCare®, ESA Inc, Chelmsford, Massachusetts, 01824, USA). All blood samples were analysed for lead content within four hours of collection or placed in buffer solution and refrigerated within the time frame recommended by the manufacturer. The analyser uses a technique known as anodic stripping voltammetry (ASV) described in Wang (2000) and has a detection range of 0.0 mg/L - 0.65 mg/L and a analytical reporting range of 0.014 mg/L – 0.65 mg/L (Anonymous, 2005). Levels greater than 0.65 mg/L are expressed as “HI”. Where the upper limit of the analyser was reached, a 1:10 dilution with saline was used to give a quantitative measure.

Heparinised blood was submitted to a commercial laboratory for haematology and plasma biochemical analysis for the range of metabolites listed in Table 2.2 between three and four days post collection. Prior to submission, samples were kept refrigerated at four degrees Celsius.

Capture, handling and sampling procedures were carried out with approval from the Massey University Animal Ethics Committee and the New Zealand Department of Conservation.

Review of Wildlife Mortality Database and Archived Samples

Huia (the New Zealand wildlife pathology database) and the Massey University pathology database were searched using the search terms “Kea”, “*Nestor*” and “*Nestor notabilis*”. Individual records were retrieved for all animals entered into both databases from 1991-2007. Records from captive kea were excluded.

Formalin-fixed tissues from seven wild kea were archived at the Institute of Veterinary and Biomedical Sciences (IVABS), Massey University. Liver was retrieved from all of the birds, and kidney from three birds. Samples were submitted to the Food and Nutrition Laboratory, Massey University for lead analysis. Acid digest was performed on each sample and they were then analysed for lead content using proprietary techniques based on inductively coupled plasma mass spectrometry (ICP-MS) as described in Dean (2005). Fresh-frozen liver and kidney samples from five cases were tested at the same laboratory using ICP-MS and five cases were tested at a commercial laboratory using proprietary methods based on colorimetric measurement of trace metals as described in Sandell (1959). Results were reported as mg/kg wet weight.

Liver and kidney tissue stored in paraffin blocks were processed routinely for histology and stained with Ziehl-Neelson (ZN) to enable examination for acid-fast intra-nuclear lead inclusion bodies.

Statistical Analysis

Statistical analyses were performed using SigmaStat© for Windows, version 3.5 (SPSS Inc., Chicago, IL 60606, USA). Normally distributed data was compared using a t-test. Data without a normal distribution was compared using a Mann-Whitney Rank Sum Test. Linear regression was performed to assess a correlation between blood lead level and selected findings. Unless otherwise indicated, means are expressed followed by the standard deviation (s.d).

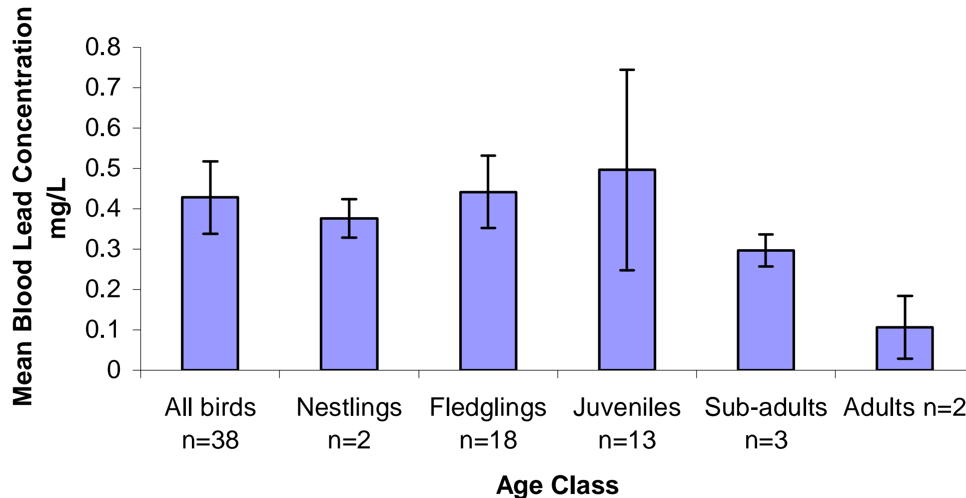
Results

Blood Lead Survey of Wild Kea

All the birds sampled (n = 38) had been exposed to lead (Figure 2.2) and 35 out of 38 (92%) had blood lead concentrations above 0.1 mg/L at the time of testing. The blood lead concentration ranged from 0.028 mg/L to 3.43 mg/L with a mean of 0.428 mg/L ± 0.581.

Eighteen of the 38 birds were fledglings and all these birds had blood lead concentrations greater than 0.1 mg/L. The blood lead concentration of the fledgling kea ranged from 0.156 mg/L to 1.7 mg/L with a mean of 0.442 mg/L ± 0.387. Thirteen of the

Figure 2.2: Mean blood lead concentrations of different age groups of kea from Mt Cook/Aoraki National Park. Error bars represent one standard error.



38 kea tested were juvenile birds with 12/13 (92%) birds having blood lead concentrations levels greater than 0.1 mg/L. The blood lead concentration of the juvenile kea ranged from 0.087mg/L to 3.43 mg/L with a mean of 0.496mg/L ± 0.894. No statistically significant differences were found in blood lead concentrations between juvenile and fledgling kea (Mann-Whitney Rank Sum; T=165, P=0.089).

Two nestling kea were blood sampled from a nest site near Mt Cook/ Aoraki Village and were found to have blood lead concentrations of 0.328 and 0.424 mg/L.

A single sub-adult female, two sub-adult male and two adult male kea were tested. Their blood lead concentrations were 0.23, 0.293, 0.368, 0.028 and 0.183 mg/L respectively. Insufficient sub-adult, adult and female birds were sampled to enable further age or sex comparisons.

None of the kea tested showed overt clinical signs at the time of sampling. A single juvenile bird (blood lead 0.247 mg/L) was observed to be ataxic and clumsy with a wide-based stance the day after sampling, however the bird was not recaptured and not seen again during the remainder of the field trip or on subsequent field trips.

Condition Score

Twelve juvenile, two sub-adult and one adult bird were examined (n = 15). All of the birds were in good to adequate condition and there was no relationship found between condition score and blood lead level ($n = 15$, $r^2 = 0.003$, $p = 0.8$)

Haematology and Biochemistry

Haematology and biochemistry parameters were obtained for 18 kea of mixed age. The mean packed cell volume (PCV) was 0.50 L/L ± 0.04 with a range 0.43-0.58 (Table 2.1). Sufficient data were not available to compare age groups or sexes. There was no significant relationship found between lead level and PCV ($n = 18$, $r^2 = 0.1$, $p = 0.2$). Estimated white cell counts (WCC) were carried out on eight of 15 blood smears. The remainder were rejected due to poor smear quality which varied due to field conditions. The mean total WCC (n = 8) was 15.6 x 10^9cells/L ± 6.2 with a range of 8.4-24.8 x 10^9cells/L.

A significant linear relationship was found by regression analysis between blood lead concentration and WCC ($r^2 = 0.66$, $p = 0.01$) with WCC increasing as blood lead level increased (Figure 2.3).

Detailed results of serum biochemistry analysis are included in Table 2.2. There were no significant abnormalities noted in serum biochemistry with the exception that total protein and globulins were lower in the wild kea (total protein mean = 26.39 ± 2.89; globulin mean = 14.06 ± 2.86) than International Species Information System (ISIS) published ranges for captive kea (total protein mean = 37 ± 7; globulin mean = 29 ± 8)(ISIS, 2002). Raw data is presented in Appendix 3.

Table 2.1: Haematology values from wild kea at Aoraki/ Mount Cook Village and National Park, collected during May and December 2006 and January-February 2007. Values reported include packed cell volume (PCV) and white cell count (WCC).

Test	**N**	**Wild kea from Mt Cook mean ± SD (range)**
PCV L/L	19	0.50 ± 0.04 (0.43-0.58)
WCC x10^9/L	8	15.59 ± 6.22 (8.4-24.8)
Heterophil %	8	74.98 ± 11.34 (58-90)
Heterophil x10^9/L	8	11.73 ±5.06 (4.9-21.1)
Lymphocyte %	8	21.38 ± 11.51 (8-38)
Lymphocyte x10^9/L	8	3.30 ± 2.49 (0.9-8.9)
Monocyte %	8	3.38 ± 2.07 (1-7)
Monocyte x10^9/L	8	0.44 ± 0.26 (0.2-1.0)
Eosinophil %	8	0
Eosinophil x10^9/L	8	0
Basophil %	8	0.38 ± 0.74 (0-2)
Basophil x10^9/L	8	0.09 ± 0.2 (0-0.5)

Figure 2.3: Regression of white cell count versus blood lead level in eight kea from Aoraki/ Mt Cook Village.

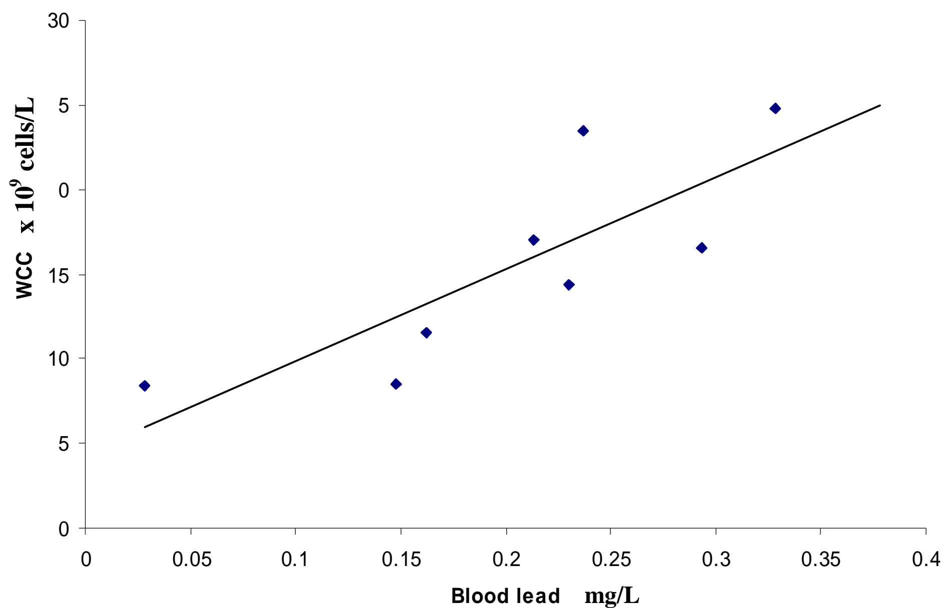


Table 2.2: Serum biochemical and electrolyte values from wild kea at Aoraki/ Mount Cook Village, collected during May and December 2006 and January-February 2007. Values reported include creatine kinase (CK), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT) and albumin/globulin ration (A/G ratio). Values are reported in SI units.

Biochemical analyte	**N**	**Wild kea from Mt Cook Mean ± SD (Range)**
Uric Acid umol/L	18	110.44 ± 86.32 (35-328)
CK IU/L 37C	18	633.67 ± 220.46 (358-1182)
AST IU/L 37C	18	152 ± 20.86 (117-195)
GGT IU/L 37C	18	0.72 ± 1.07 (0-3)
Total Protein g/L	18	26.39 ± 2.89 (21-33)
Albumin g/L	18	12.33 ± 1.41 (10-15)
Globulins g/L	18	14.06 ± 2.86 (10-23)
A/G ratio	18	0.91 ± 0.18 (0.43-1.18)
Calcium mmol/L	18	2.14 ± 0.12 (1.93-2.44)
Glucose mmol/L	18	15.6 ± 3.70 (10.8-23.9)
Sodium mmol/L	13	145.15 ± 3.53 (141-153)
Potassium mmol/L	13	4.40 ± 2.40 (2.2-10.6)
Chloride mmol/L	13	112.58 ±1.83 (110-116)

Review of Wildlife Mortality Database

From 1991-2007 there have been 15 wild kea submitted to Massey University for diagnostic pathology. Seven (47%) of the dead wild birds were adults, three (20%) were juveniles, two (13%) were sub-adults, two (13%) were fledglings and age was unrecorded for one (7%) bird. Of the wild birds submitted, 7/15 (47%) were confirmed with elevated liver and/or kidney lead concentrations, 6/15 (40%) did not have elevated tissue lead concentrations and 13% (two) were not tested. Two of the seven adults (29%), both sub-adults, two of three juveniles (67%) and one of two fledglings (50%) had elevated tissue lead consistent with possible toxicosis. All the birds with elevated tissue lead concentrations (n = 7) were seen exhibiting abnormal clinical signs prior to death (Table 2.3). Gross pathology was not consistent between birds. ZN positive intra-nuclear inclusions were found in the renal tubular epithelium in 100% (7) of cases with elevated

Table 2.3: Clinical signs noted prior to death in wild kea with elevated liver, kidney and blood lead concentrations. Kea are listed in order of decreasing liver lead levels

Kea number	Clinical Signs					
	Regurgitation	**Weak/ Recumbent**	**Ataxia/ seizures**	**Poor body condition**	**Diarrhoea**	**Dyspnoea**
1	+	+	-	+	+	-
3	+	-	-	+	-	-
4	-	-	-	+	-	-
6	-	+	+	+	+	-
5	+	-	-	+	-	-
2	-	+	+	-	-	+
7*	?	?	?	+	?	?

* This bird was observed to be "ill" prior to death. Tissue lead levels were recorded as elevated but values were not included in necropsy report.

tissue lead and in hepatocytes in two cases (Figure 2.4) . Histopathological findings in the kea with elevated tissue lead concentrations are presented in Table 2.4.

Analysis of formalin-fixed tissue found one bird with elevated lead tissue levels that had previously not been tested. Due to small sample size, statistical analysis of lead levels in fresh compared with formalin fixed tissue has not been attempted. However there is an observed variation between tissue lead concentrations between fresh and formalin-fixed tissue (Table 2.5).

Table 2.4: Histopathological lesions in wild kea with elevated liver and renal lead concentrations. Kea are listed in order of decreasing liver lead levels.

Kea number	Histopathological Lesions			
	Renal tubular degeneration	**Acid-fast intranuclear inclusions in renal cells**	**Acid-fast intranuclear inclusions in hepatic cells**	**Hepatocellular degeneration**
1	+	+	-	+
3	-	+	-	-
4	-	+	-	-
6	-	+	+	-
5	-	+	-	-
2	+	+	+	-
7*	-	+	-	-

* Tissue lead levels were recorded as elevated but values were not included in necropsy report.

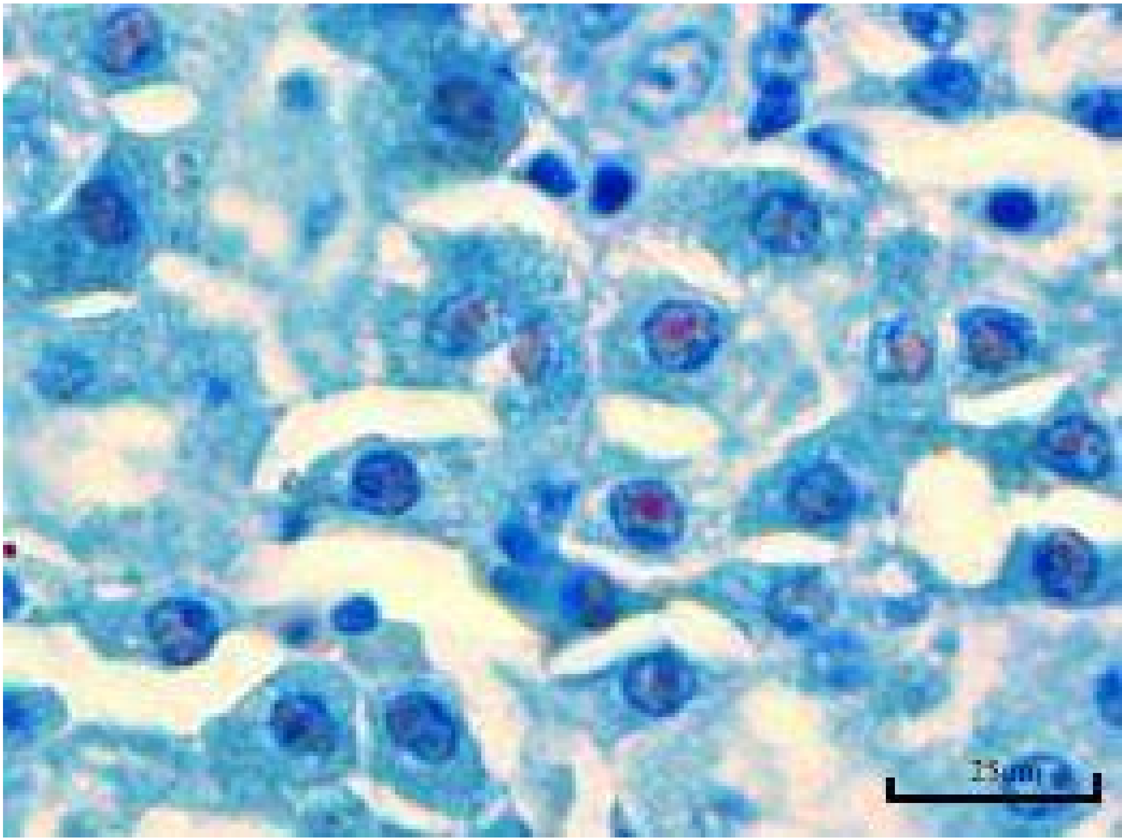
Table 2.5: Comparison of liver and kidney lead levels in fresh-frozen and formalin-fixed (FF) tissue (n=6).

Kea number	Fresh-frozen liver mg/kg ww	Formalin-fixed liver mg/kg ww	Fresh-frozen kidney mg/kg ww	Formalin-fixed kidney mg/kg ww
1	57.2[a]	80[b]	-	-
2	10[a]	6.8[b]	-	-
3	28.2[a]	81[b]	-	230[b]
4	-	54[b]	-	160[b]
5	13.4[a]	-	15.2[a]	-
6	15[b]	-	27[b]	-

[a] Analysis performed by colorimetric analysis

[b] Analysis performed by ICP-MS

Figure 2.4: Photomicrograph of ZN positive intra-nuclear inclusions in a lead exposed kea.



Discussion:

It is clearly demonstrated in this study that lead is present in all the kea surveyed and may be an important contributing factor to mortality in the wild population. Most fledglings were caught outside the village away from the buildings and have only recently left the nest while juveniles were caught in the village and were observed chewing on the buildings. Despite this, fledging kea were found to have blood lead concentration within the same range as juvenile birds. Fledgling kea are fed by adults after leaving the nest until they learn to forage for themselves (Diamond and Bond, 1999) suggesting that fledgling lead exposure may be from a combination of parent feeding and self exposure.

Two nestlings sampled were also found to have elevated blood lead levels within the same range as the free-ranging birds. It is probable that exposure to lead begins in the nest with adults feeding their young lead-containing material. It is also possible that lead is transferred to kea nestlings via the egg when the adult female has been previously exposed to lead (Burger and Gochfeld, 1993). However blood lead concentrations are representative of recent exposure and bone lead and egg-shell lead would need to be examined to explore this further. During reproduction it is possible that blood lead concentration of previously exposed females may increase from lead released from bone. Wilson et al (2007) found blood-lead concentrations of reproductively active Pacific common eiders (*Somateria mollisima v-nigrum)* increased significantly above levels in non-reproductive birds.

None of the wild birds examined showed overt clinical signs of lead intoxication (such as regurgitation or neurological disease) at the time of testing, however one bird did appear ataxic the day after sampling, possibly due to lead exposure. General physical examination of juvenile birds revealed few abnormalities with poor feather condition being the most common finding. This is most likely explained by normal wear and tear on the feathers as kea do not moult out of their fledgling plumage until their second summer (Diamond and Bond, 1999).

Biochemistry and red blood cell parameters were unremarkable, in contrast to Jarrett (1998), who found a significant negative correlation between blood-lead levels and haemoglobin ($p < 0.001$), PCV ($p < 0.05$) and serum albumin ($p = 0.05$). However, Jarrett (1998) also found that there was no significant difference between PCV and haemoglobin

levels of zoo and wild kea. Blood-lead levels of the zoo kea sampled in this study were not reported making it difficult to compare the two populations.

White cell count was found to be positively correlated with the blood-lead level in juvenile birds (Figure 2.3) suggesting that elevated lead levels may be having an effect on the health of the birds, however the sample size is small (n = 8) and the quality of the smears were poor making it difficult to draw conclusions as to the significance of this. Further, blood smears from fledglings were not examined and there are no published reference values for blood parameters in wild kea. However, sub-clinical lead poisoning is known to increase an animal's susceptibility to disease (Pattee and Pain, 2003) and to cause immune system disruption (Dietert et al., 2004).

In captive parrots, it has been suggested that blood lead levels greater than 0.2 mg/L are suggestive of lead poisoning and if accompanied by clinical signs, levels greater than 0.4-0.6 mg/L are diagnostic (Dumonceaux and Harrison, 1994). However, some birds have shown clinical signs and responded to therapy with blood lead levels as low as 0.1mg/L, and others have had no clinical signs with much higher levels (Dumonceaux and Harrison, 1994). While the absence of observed clinical signs in the wild kea sampled in this study may suggest tolerance to high blood lead concentrations, this does not take into account the sub-clinical effects of low-level exposure. A paradigm shift in human medicine from concentrating on symptomatic patients to a focus on sub-clinical effects of low-level lead exposure has occurred over the last 30 years (Needleman, 2004). The effects of lead on the development of neurological function and intelligence in people has been well studied (Needleman, 2004). In humans, low-level chronic lead exposure is known to cause impaired learning ability, neuromuscular defects and altered haeme metabolism (Silbergeld and Goldberg, 1980) when present at levels less than that causing clinical signs with some authors suggesting levels less than 0.1 mg/L may also have a significant effect (Needleman, 2004; Gilbert and Weiss, 2006). Gilbert and Weiss (2006) suggest that blood lead levels greater than 0.02mg/L should be regarded as elevated due to the effects of even very low levels of lead on biological processes, particularly the nervous system. The effect of lead exposure on herring gull (*Larus argentatus)* behaviour in the wild and in the laboratory has been extensively studied by Burger and Gochfeld (e.g. Burger and Gochfeld, 2005). They found that low levels of lead affected neurobehavioral development and

learning and while each behavioural effect (such as reduced begging and reduced thermoregulatory ability) may be small, cumulatively they may result in reduced survival (Burger and Gochfeld, 2005).

Oral exploratory behaviour is commonly exhibited in young animals and extensive mouthing behaviour can lead to an increased risk of lead ingestion (Berney, 1996; Needleman, 2004). Young kea exhibit exploratory behaviour similar to young humans (Temple, 1996). Fledglings emerge without an innate knowledge of how to distinguish edible from inedible foods and start by manipulating and chewing on any object they encounter (Diamond and Bond, 1999). During their first two years, kea spend considerable time developing motor and coordination skills as well as feeding and foraging skills which are important for survival in a sub-alpine environment (Temple, 1996). They will forage in areas with easily accessible food such as rubbish dumps and towns (Diamond and Bond, 1999). This is likely to increase their risk of developing lead toxicosis. This hypothesis is supported by our findings with most juvenile and all fledgling kea sampled exhibiting lead levels greater than 0.1 mg/L. Long term studies of children and young gulls have shown that the effects of lead exposure early in life can persist into at least early adulthood and can result in a range of neurological disorders and deficits such as learning disabilities, and problems with fine motor function (Rubin and Farber, 1994; Anderson et al., 1996; Needleman, 2004; Burger and Gochfeld, 2005). Our findings raise the possibility that similar effects on learning and intelligence may be occurring in kea.

All the kea sampled were captured in areas near human habitation and 35 of the 38 birds sampled (92%) were male. In studies of kea at a rubbish dump, it was found that more male kea are seen (Bond and Diamond, 1992). Young male birds may be at increased risk as they commonly travel further from their nesting site than female birds and they frequently group together to feed (Peat, 1995; Diamond and Bond, 1999). These groups are often found in human inhabited areas and it is in these areas where toxic substances are most available. Fewer female birds were seen during the duration of the study and those present were more cautious than male birds and were less likely to be caught. Adult birds were also seen less frequently and were more difficult to catch. Further investigation of lead levels in sub-adult kea, adult kea, and female kea is warranted for comparison.

Lead has indiscriminate effects on many body systems which can lead to a wide range of non-specific pathological changes. Low-level chronic exposure may cause different pathology than higher level acute exposure. Inter- and intra-species variation in the uptake, distribution, toxic effects and elimination of lead (Silbergeld and Goldberg, 1980; Pattee and Pain, 2003) may result in a range of pathological changes and pathology in wild birds can be particularly variable as there may be differing times from exposure to death and from death to submission of the specimen. It is important to distinguish lead poisoning as a cause of mortality from lead exposure (Franson, 1996) and interpreting tissue lead levels in the absence of clinical and pathological changes is difficult, particularly without published reference ranges for tissue lead levels. Franson (1996) suggests making an informed diagnosis based on clinical and pathological findings combined with tissue residues. In this study, all of the necropsied kea that had elevated tissue lead levels were observed displaying signs consistent with lead toxicity prior to death and all had ZN positive intranuclear inclusions in the renal tubular epithelium consistent with lead toxicosis, however no other characteristic pattern was observed.

Lead is recognized as a common nephrotoxin in domestic animals (Confer and Panciera, 1995). Lead induced cell membrane or mitochondrial damage results in acute tubular necrosis and acid-fast lead-protein complexes may form within the nucleus of tubular epithelial cells (Confer and Panciera, 1995). Histological demonstration of these inclusions is considered consistent with lead toxicosis (Confer and Panciera, 1995). Our review of renal histopathology showed evidence of renal tubular damage in some kea with elevated tissue lead levels. Acid-fast intra-nuclear inclusions were seen in all kea with elevated tissue lead. While acid -fast renal tubular intra-nuclear inclusions can occasionally be seen without elevated lead in dogs (Maxie and Youssef, 2007), to our knowledge, this has not been reported in avian species. We conclude that the presence of acid fast nuclear inclusions in the renal tubular epithelium of the kea is consistent with lead intoxication.

The tissue lead results in this study are presented as wet weight, making comparison between fresh and formalin-fixed tissue difficult due to variation in the water content of the tissues. Formalin fixed tissues are dehydrated and tissue water concentration can also vary between tissue types and between individuals (Bush et al., 1995). Bush *et al* (1995) found no difference in heavy metal concentrations between fresh and formalin-fixed human tissue

samples when tissues were compared using the same metal detection technique and presented in dry weight. However a study of lead in raccoon tissues found lead levels reported as wet weight varied between frozen and formalin fixed tissues (Hamir et al., 1995). In the avian literature, tissue lead levels are commonly reported as wet weight (Pain, 1996), however dry weight is considered more accurate due to inconsistent weight loss in tissue through drying during handling and freezing (Adrian and Stevens, 1979). Archived tissues can be useful for retrospective studies however interpretation of results can be difficult. Further work comparing heavy metal levels in fresh and formalin-fixed tissues of birds is required.

Jarrett (1998) measured the liver lead concentration of 16 wild kea and reported range of less than 0.15 mg/kg to 40.5 mg/kg (mean = 7.37 ± 12.39) with three birds thought to be clinically affected by lead (although one of these was shot). It was not reported if these results were measured in wet or dry weight.

There are many potential sources of lead in the kea's environment. Kea have been reported as causing damage to tents, ski lifts, vehicles and buildings including destruction and consumption of materials such as painted surfaces, lead flashings, aerials, and lead-head nails (the softer lead head is prised off) (Peat, 1995). Rubbish, particularly in open dumping sites as well as that left in the environment also poses a risk with access to items containing lead (such as lead-acid batteries), as well as other threats such as plastic, chocolate and other toxic industrial and household items. Lead flashing and lead-head nails are still present on buildings within the Aoraki/Mt Cook Village and nearby national park buildings and are a potential risk to kea.

This study has demonstrated a severe anthropogenic health problem affecting the kea in Aoraki/Mt Cook National Park and likely to be affecting kea populations in multiple locations. The potential effects of lead on intelligence and learning ability, as well as other body systems may be detrimental to the survival of the species. Our study examines a single population and further work to investigate the exposure to lead of different age groups of kea at this and other locations is underway. Further studies examining the effect of lead on life history traits such as reproduction, recruitment and survival as well as examination of more subtle behavioural effects will enable a better assessment of the

population effects of lead on kea. The examination of bone lead levels along with tissue levels of necropsied kea may also provide information regarding the extent of lifetime lead exposure in kea. Banding each bird captured with a unique colour band combination and number will enable identification of individual birds if repeat sampling is carried out. Lead abatement is also to be initiated in the Aoraki/ Mt Cook Village and national park buildings to reduce the exposure of kea to lead.

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Chapter 3

Lead Exposure in Wild Populations of the Critically Endangered Takahe (*Porphyrio hochstetteri*)

Abstract

Takahe *(Porphyrio hochstetteri)* are critically endangered large flightless rails, endemic to New Zealand. Forty-five takahe from three offshore islands (Mana, Tiritiri Matangi and Kapiti) were examined and had blood collected for lead analysis. Eighteen (40%) of the takahe were found to have detectable blood lead concentrations ranging from 0.015 mg/L to 0.148 mg/L (mean = 0.028 mg/L ± 0.042). Blood lead concentrations of takahe were significantly higher on Tiritiri Matangi Island ($p = 0.005$) compared to Mana Island. On Tiritiri Matangi island, a family group including a chick living around buildings was found to have significantly higher blood lead concentrations than a family group with a chick living away from buildings ($p = 0.002$). Analysis of liver and kidney samples from offshore island and Murchison Mountains birds found that all samples had detectable lead and that samples from island birds contain significantly more lead than samples from Murchison Mountain birds ($p = 0.035$), however care is required when interpreting these results due to differences in storage and fixation. This study found that despite levels of lead exposure in the population being low and unlikely to result in overt clinical signs, it is widespread and there may be significant exposure of birds living around old buildings.

Keywords

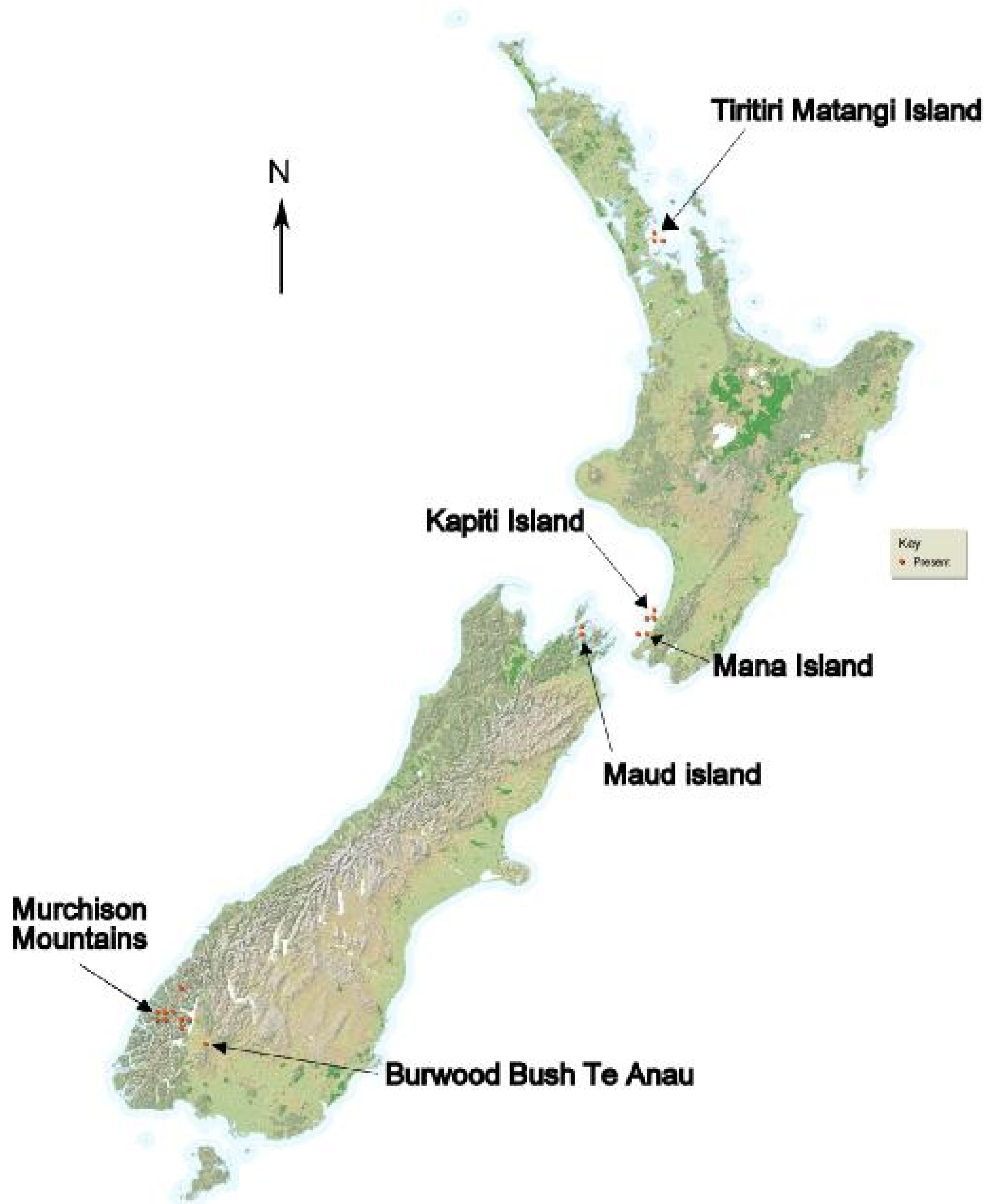
Takahe; *Porphyrio hochstetteri*; blood lead; Pb; toxicity; endangered species.

Introduction

The takahe (*Porphyrio hochstetteri*) is a large flightless rail endemic to the South Island of New Zealand. The North Island takahe (*P. mantelli*) is extinct and known only from fossils. The South Island takahe (hereafter referred to as takahe) were thought to be extinct until 250-300 birds were rediscovered in 1948 in Fiordland in the southwest of the South Island of New Zealand. Takahe historically occurred throughout forest and grass ecosystems but are now restricted to alpine tussock grasslands in the Murchison Mountains in Fiordland, and on predator free offshore islands and mainland islands where they have been introduced (Figure 3.1) (Bird Life International, 2006). Since re-discovery, the natural population has fluctuated, and has been as low as 121 birds (Maxwell, 2001). However, following intensive management, including captive breeding and the establishment of populations on four offshore island sanctuaries (Maud, Mana, Kapiti and Tiritiri Matangi Islands), the current population (excluding display birds) is approximately 217 (Phil Tisch,Linda Kilduff, DOC, 2008). This is mainly due to growth in the offshore island populations (Jamieson and Ryan, 2001). Takahe are listed as endangered by the World Conservation Union (IUCN) red list (BirdLife_International, 2006) and as nationally critical under the New Zealand Threat Classification List (Hitchmough et al., 2007).

Offshore island populations of takahe are managed as an insurance population against the decline of the naturally occurring Fiordland population (Crouchley, 1994), and loss of genetic diversity is minimised by treating these birds as one population and selectively translocating birds between offshore islands, however inbreeding depression potentially resulting in decreased egg fertility is a concern (Jamieson et al., 2006). The offshore islands are outside the natural range of the takahe and have historically been inhabited by humans and used for farming and lighthouse keeping. Old buildings still exist on these islands and are maintained for conservation use and historical significance. On Tiritiri Matangi Island there is an operational lighthouse. There are small numbers of conservation staff permanently residing on all of the islands with takahe populations and Kapiti Island has a small community on it. Tourism is permitted on all four islands; however, visitor numbers are restricted on Kapiti, Mana, and particularly, Maud Islands. Takahe on offshore islands are free from the effects of introduced predators and competitors and experience milder weather than the Fiordland population.

Figure 3.1: Distribution of takahe in New Zealand showing location of study site (modified from Robertson *et al* (2007)



Despite these advantages resulting in higher adult survival rates, takahe from offshore island populations have been shown to have lower egg fertility and raise fewer juveniles per egg (Jamieson and Ryan, 2001; Jamieson et al., 2006). Jamieson and Ryan (2001) concluded that this was largely due to inbreeding depression based on the

assumption that “no specific environmental variables that can be directly linked to the poor fertility and hatchability of offshore island takahe eggs have been identified”.

Two takahe from Kapiti Island treated at the Wildlife Ward of the New Zealand Wildlife Health Centre, Massey University, were diagnosed with elevated blood lead levels in addition to other problems. Lead is a highly toxic, non-essential metal that can have effects on many body systems, even at very low levels (Gilbert and Weiss, 2006). Chronic, low-level exposure can cause sub-clinical disease of the nervous system and reproductive system and can cause immune dysfunction (Pattee and Pain, 2003; Dietert et al., 2004). It is possible that previous land use of the offshore islands may have increased the environmental lead available to takahe. Sources of lead may include lead shot and lead paint and may be a concern in reintroduction and translocation programs. Lead may also be ingested with soil while feeding and indirectly via invertebrates such as worms which can concentrate lead if living in contaminated soil (Darling and Thomas, 2005). Takahe foraging habits may predispose these birds to lead ingestion leading to acute poisonings, manifesting as sudden disease with obvious neurological signs, or chronic exposure resulting in more long term and insidious sub-clinical effects. Lead exposure may be a threat to the offshore island population of takahe.

This study aimed to examine lead exposure in takahe by:

1. surveying the blood lead concentrations in takahe on Tiritiri Matangi, Kapiti and Mana Islands; and
2. analysing tissue lead concentrations in necropsy specimens from offshore island and Fiordland takahe populations.

Materials and Methods

Study Location

Takahe from three New Zealand offshore islands were examined in this study. Mana Island (41°08’66”S, 174°78’54”E) and Kapiti Island (40°85’02”S, 174°92’14”E) are located off the west coast of the North Island of New Zealand (Figure 1). Tiritiri Matangi Island (36°60’63”S, 174°89’50”) is located in the Hauraki Gulf close to Auckland in the North Island of New Zealand (Figure 3.1). All three offshore islands were cleared and farmed prior to becoming protected areas. Conservation staff

permanently reside on all three offshore islands. Levels of tourism and supporting infrastructure varies between offshore islands with very few tourists visiting Mana Island and less than 50 visitors per day on Kapiti Island, while Tiritiri Matangi has greater than 100 tourists daily.

Sample Collection and Analysis

Forty-five takahe were sampled during 2006. On Mana Island 24 of the 43 resident takahe were sampled in May 2006 (autumn). On Tiritiri Matangi Island 13 of the 16 resident birds were sampled in August 2006 (winter) and on Kapiti Island eight of the12 takahe were sampled in September (spring) 2006. The takahe were captured by hand, handnet or by corralling in family groups, using a food lure. Food-based training is in place with many of the takahe to facilitate less stressful capture.

Birds were identified by known location, colour band combinations and uniquely numbered leg bands. Blood was sampled from the left or right medial metatarsal vein. Two smears for microscopy were made with fresh blood and the remaining blood was placed into 0.4 ml lithium heparin microtainers (Becton Dickenson Vacutainer Systems, Preanalytical Solutions, Franklin Lakes, NJ 07417).

To further characterize the differences in blood lead concentrations between birds and between offshore islands, we recorded whether birds were part of a family group raising a chick and whether their territory included a building.

A clinical examination was carried out on each bird. The condition of the bird was estimated using a combination of the prominence of the keel and the pectoral muscles and the presence of subcutaneous fat (Doneley et al., 2006) as well as the prominence of the dorsal pelvic region (by palpating over the pelvic area). Each bird was examined by the author to ensure consistency. Morphometric measurements and a blood feather for DNA sexing were collected from each chick. Birds were banded following sample collection. All birds on Mana and Tiritiri Matangi Islands and two birds on Kapiti Island were weighed prior to release. Following release, the takahe were fed a small handful of pellets as positive reinforcement.

Blood lead concentration was measured in the field using a portable lead analyser (Leadcare®, ESA Inc, Chelmsford, Massachusetts, 01824, USA). All blood samples were analysed for lead content within three hours of collection. The analyser uses a technique known as anodic stripping voltammetry (ASV) described by Wang

(2000) and has a detection range of 0.0 mg/l -0.65 mg/l and a analytical reporting range of 0.014 mg/l – 0.65 mg/l (Anonymous, 2005).

Packed cell volume (PCV) was measured using 100 μL microhaematocrit tubes and a microhaematocrit centrifuge MK5 (Hawksley and Sons Ltd, Malborough Rd, Lancing, Sussex, England). Heparinised blood was spun for five minutes at 4000 RPM and the plasma harvested and refrigerated. Plasma samples were submitted to a commercial laboratory for biochemical analysis of uric acid, creatine kinase (CK), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), total protein, albumin, globulin, albumin/globulin (A/G) ratio, calcium, glucose, sodium, potassium, chloride and bile acids between two and four days post collection. Prior to submission, plasma samples were refrigerated at four degrees Celsius.

All procedures were carried out with permission from the Massey University Animal Ethics Committee and the New Zealand Department of Conservation.

Lead Analysis of Tissue Samples

Archived tissue preserved in 10% neutral buffered formalin from 22 wild offshore island takahe was retrieved. Between 0.6 gm and 2.5 gm of formalin preserved kidney tissue from 21 birds and liver tissue from one bird were collected. Frozen tissue was harvested from 25 dead birds from the wild population in the Murchison Mountains. These bodies were in varying states of decomposition, and had been stored frozen at -20 ° C (-4 ºF) for up to 15 years in a Department of Conservation facility. Twenty kidney and seven liver samples were collected. Samples were submitted to the Massey University Institute of Food, Nutrition and Human Health laboratory for lead analysis. Samples were subjected to acid digest and then lead analysis was performed using proprietary techniques based on inductively coupled plasma mass spectrometry (ICP-MS) as described in Dean (2005). The ICP-MS limit of detection was 0.002 mg/kg. Results were reported as mg/kg wet weight.

Statistical Analysis

Statistical analyses were performed using SigmaStat© for Windows, version 3.5 (SPSS Inc., Chicago, IL 60606, USA). Normally distributed data was compared using an analysis of variance (ANOVA). Data without a normal distribution was compared using a Kruskal-Wallis one-way analysis of variance on ranks. When differences were

identified, a pairwise comparison was performed using the Holm-Sidak method on normally distributed data and the Dunn's method on data without a normal distribution.

Linear regression was performed to determine if a correlation existed between blood lead level and selected clinical measurements. For the purposes of statistical analysis, blood lead levels below the analytical reporting range of the analyser have been reported as 0.014 mg/L. Unless otherwise specified, results are presented as mean ± standard deviation.

Results

Blood Lead Survey of Takahe

Eighteen of the 45 takahe tested (40%) had blood lead levels greater than 0.014 mg/L ranging from 0.015 mg/L to 0.148 mg/L with a mean of 0.028 mg/L ± 0.042. Four of these takahe (9%) had blood lead levels greater than 0.1 mg/L (0.107 mg/L, 0.111 mg/L, 0.116 mg/L, 0.148 mg/L). Twenty seven of the 45 takahe (60%) had lead levels below the analytical reporting level of the analyser (< 0.014 mg/L). Clinical signs of lead toxicity were not observed in any of the birds examined in this study.

Comparison of blood lead levels between offshore islands

There was a significant difference in the concentration of blood lead between takahe on different offshore islands (Kruskal-Wallis: $H = 10.648$, $df = 2$, $p = 0.005$) (Figure 3.2). Posthoc analysis showed that the blood lead concentration of takahe on Tiritiri Matangi island differed significantly from those on Mana Island (Dunn's: $Q = 2.666$, $p < 0.05$). The mean blood lead concentration of takahe on Mana Island was 0.02 mg/L ± 0.022 (range < 0.0l4 – 0.116 mg/L); in takahe on Tiritiri Matangi Island was 0.045 mg/L ± 0.047 (range < 0.014 – 0.148 mg/L); and in takahe on Kapiti Island was 0.028 mg/L ± 0.03 (range < 0.014 - 0.032 mg/L) (Figure 3.2). Nine of the 17 (69 %) birds sampled on Tiritiri Matangi had blood lead concentrations within the analytical reporting range of the analyser; compared to four of 24 (17%) takahe sampled on Mana Island. Five of the eight birds tested on Kapiti Island (62.5%) had blood lead concentrations within the analytical reporting range of the analyser. One bird from Mana Island (4%); three birds from Tiritiri Matangi Island (23 %) and no birds from Kapiti Island had blood lead concentrations greater than 0.1 mg/L (Figure 3.3).

Figure 3.2: Mean Blood Lead Levels of Takahe from Mana, Tiritiri Matangi and Kapiti Islands. The error bars represent the standard error.

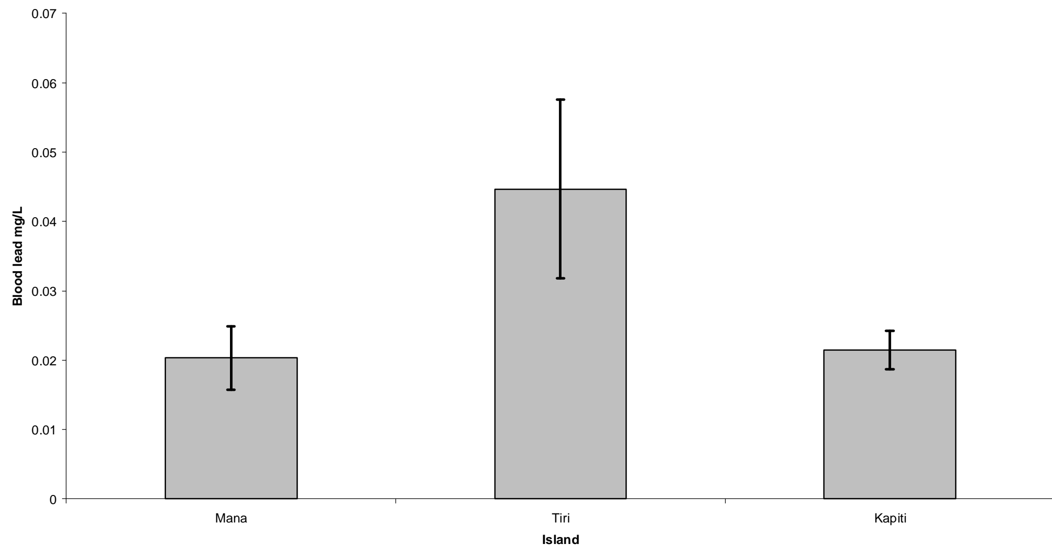
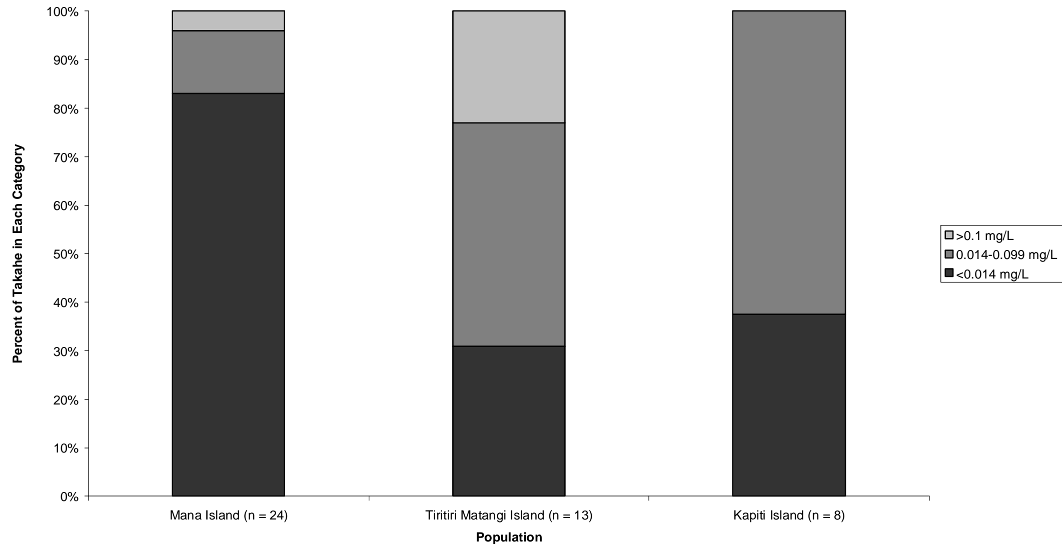


Figure 3.3: Proportion of takahe from from Mana, Tiritiri Matangi and Kapiti Islands with blood lead levels within each category (< 0.014 mg/L, 0.014-0.099 mg/L and >0.1mg/L)



Blood lead levels of family groups raising chicks with and without buildings in their range.

On Tiritiri Matangi Island, birds in a family group raising a chick around buildings (n = 3) were found to have significantly higher blood lead concentrations (mean = 0.122 mg/L ± 0.023) than a family group raising a chick away from buildings

(n = 3; mean = 0.0190 mg/L ± 0.0078) (ANOVA; F = 55.64, p = 0.002). However, on Mana Island, birds in family groups raising chicks around buildings (n=3) did not have significantly higher blood lead concentrations than those raising chicks away from buildings (n=11) (Kruskal-Wallis; H = 1.322, df = 1, p = 0.250).

Blood lead levels of takahe with and without buildings in their territory

On Mana Island, the blood lead levels of all takahe regardless of reproductive status living near buildings (mean = 0.027 mg/L ± 0.036) was higher than those living away from buildings (mean =0.017 mg/L ± 0.012), however this was not found to be significant (Kruskal-Wallis; H = 3.381, df = 1, p = 0.066). On Tiritiri Matangi Island the mean blood lead levels of birds living in proximity to buildings was 0.061 mg/L ± 0.054 and those living away from buildings was 0.018 mg/L ± 0.006, however a statistically significant difference was not detected (Kruskal-Wallis; H = 1.596, df = 1, p = 0.222).

No other significant effects were identified. These variables were not analysed for takahe from Kapiti Island as there were no family groups with chicks and 88 % (7/8) of the birds lived around buildings.

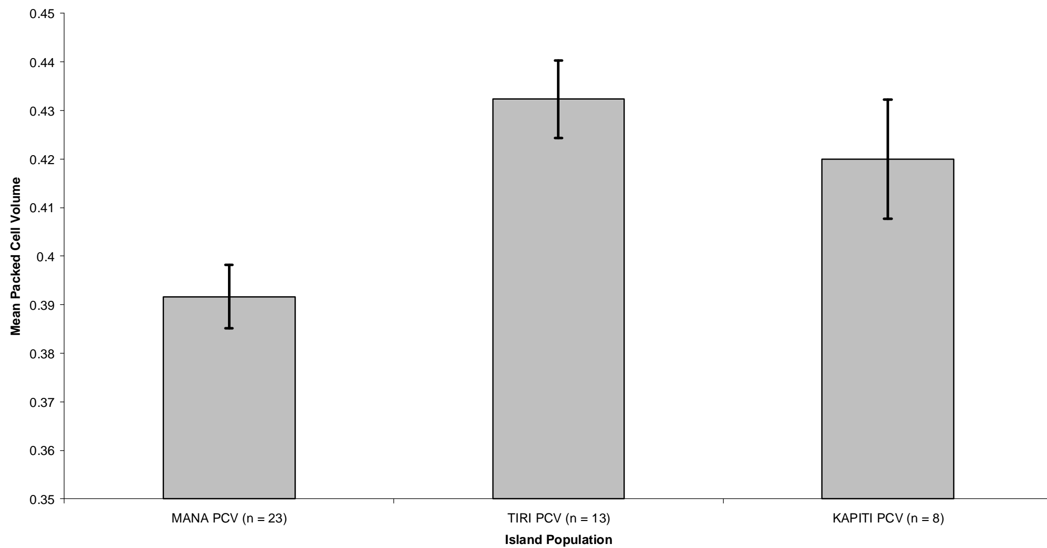
Other variables

No significant difference was found between blood lead concentrations of adult male and female takahe on any offshore island. No significant differences were found in body condition score (Kruskal-Wallis; H = 3.793, p = 0.15) or weight (ANOVA; F = 1.34, P = 0.27) of takahe from each of the three offshore islands. Body condition score and body weight did not correlate with blood lead concentrations.

PCV differed significantly between offshore islands (ANOVA; F = 8.154, P = 0.001) but did not correlate to blood lead concentration (Figure 3.4). Posthoc analysis showed that the PCV of takahe from Mana Island differed from that of birds on both Tiritiri Matangi (Holms-Sidak; t = 3.8, p = 0.0005) and Kapiti Islands (Holms-Sidak; t = 2.432, p = 0.0194).

There were no significant abnormalities noted in serum biochemistry, however statistically significant differences in multiple analytes were identified between offshore islands. (Appendix 4) These did not correlate to lead and are therefore not examined further in this paper.

Figure 3.4: Mean packed cell volume (PCV) of takahe from Mana, Tiritiri Matangi and Kapiti Islands. The error bars represent the standard error.



Lead Analysis of Archived Tissue

Lead was detected in all the tissue samples analysed (Table 3.1). Offshore island populations of takahe had significantly higher tissue lead concentrations than birds from the Murchison Mountains (Kruskal-Wallis; $H = 4.456$, $p = 0.035$). Raw data is presented in Appendix 5.

Frozen tissue

There was no significant difference between sexes in kidney lead concentrations (Kruskal-Wallis; $H = 1.32$, $p = 0.251$) or liver lead concentrations (Kruskal-Wallis; $H = 0.857$, $p = 0.533$) of adult birds. One adult female had a liver lead level of 3.9 mg/kg.

Formalin-fixed tissue

No significant difference was found between tissue lead levels from birds originating from different offshore islands (Kruskal-Wallis; $H = 7.293$, $p = 0.063$).

Table 3.1: Tissue lead concentrations from Takahe. Mean ± Standard deviation (Range)

Tissue type	Tissue lead levels (mg/kg)
Offshore island birds kidney (n=20)	0.12 ± 0.17 (0.008-2.31)
Offshore island birds liver (n=1)	0.18
Murchison birds kidney (n=20)	0.0592 ± 0.0815 (0.0087-0.36)
Murchison birds liver n= 7	0.6 ± 1.45 (0.28-3.9)

Discussion

This study conclusively demonstrates that takahe from offshore island populations and the wild populations in the Murchison Mountains are exposed to environmental lead. What is less certain is the biological effect of these lead concentrations on the health and reproductive success of takahe. Very low concentrations of lead are known to affect biological systems (Pain, 1996) and increased susceptibility to predation, starvation and infection may occur due to lead-induced physiological and behavioural changes (Fisher et al., 2006). Lead exposure has been reported to cause reproductive abnormalities in wildlife (Hoffman, 2003). In mammals, lead exposure has been correlated to impaired spermatogenesis, sperm motility and effects on embryo development (Winder, 1993; Gross et al., 2003). Our study provides no direct evidence that lead is affecting the fertility of takahe, but further studies are necessary. Direct and indirect effects on the central nervous system, particularly in young animals can lead to developmental changes resulting in learning disabilities that may affect survival (Burger and Gochfeld, 1997; Burger and Gochfeld, 2000; Burger and Gochfeld, 2005). Immune system disruption can occur at low levels of lead exposure, making an animal more susceptible to disease (Hemphill et al., 1971; Schlick and Friedberg, 1981; Trust et al., 1990). Reduced lifespan and increased mortality has also been identified as a consequence of lead exposure in humans (Dart et al., 2004; Needleman, 2004) and may be a concern for longer lived species such as takahe.

The offshore island populations of takahe are managed as a single population via occasional translocations of birds based on population breeding requirements (Crouchley, 1994), however differences were found between the blood lead concentrations of birds living on Mana Island and Tiritiri Matangi Island. Blood lead concentration usually represents recent exposure and is therefore likely to be

representative of lead acquired from the island of residence at the time of sampling (although lead mobilized from bone may also increase blood lead concentrations). This suggests that lead contamination of the environment and the availability of lead for ingestion is potentially greater on Tiritiri Matangi Island than on Mana Island. All of the offshore islands contain highly modified environments with histories including significant clearing, farming and lighthouse keeping. On Tiritiri Matangi Island there is an operational lighthouse built in the 1800s, cottages built in the early 1900s that are now used as a rangers residence and research house as well as supporting infrastructure (such as machinery sheds). The lighthouse on Mana Island was removed in the late 1800s however other buildings still remain for historic and conservation purposes. Kapiti Island also contains several old houses used for volunteers and rangers, and also supports a small community of people.

Despite restriction in the use of lead paint (NZ Toxic Substances Regulation Act 1983), older buildings with leaded paint are still considered a significant source of lead. The old buildings present on the study offshore islands potentially still contain aged lead paint that is flaking off causing local contamination of the soil around the buildings. Larger flakes may also be ingested and have been recorded as causing morbidity and mortality of Laysan albatross (*Diomeda ummutablis)* chicks on Midway Atoll, Hawaii (Sileo and Fefer, 1987).

On Mana Island, blood lead concentrations of birds living near buildings were found to be higher than those living away from buildings, although this was not found to be statistically significant ($p = 0.066$). However, our sample size was necessarily restricted by the low number of birds available and an increased sample size may have confirmed the observed difference. Human-induced lead contamination is often unevenly distributed in the environment depending on land use and placement of buildings, and therefore differences in exposure based on territory location would be expected. This was not found to be the case on Tiritiri Matangi Island, however more birds live near buildings and a higher proportion of the population were found to have elevated blood lead concentrations on this island. On Tiritiri Matangi Island, significantly higher blood lead concentrations were detected in a family group living around buildings than in a family group living away from buildings ($p = 0.002$). However the effect of proximity to buildings is not observed when all the takahe sampled on Tiritiri Matangi Island are examined. Despite the low number of birds in

each family (one male, one female and one chick), this finding does suggest that lead contamination of the environment from buildings may be occurring.

While no significant difference between blood lead concentrations of birds in a family group raising a chick and those without a chick was detected in this study, it is still possible that raising a chick may increase exposure to lead. Takahe raising chicks feed differently to non-reproductive takahe by pulling up clumps of vegetation and ingesting soil and invertebrates as well as plant material rather than just cropping grass or pulling up tillers (Lee and Jamieson, 2001). Soil is recognised as a potential source of toxin exposure for wildlife (Beyer et al., 1994). Soil may be intentionally ingested or ingested while preening or feeding on plants or invertebrates (Beyer et al., 1994; Beyer and Fries, 2003). During this study, a takahe parent was observed to be pulling up clumps of grass and offering earthworms to the chick. Earthworms act as bioaccumulators of metals (Reinecke et al., 2000; Booth et al., 2003; Darling and Thomas, 2005) and animals that feed on earthworms may obtain toxic levels of lead from this food source (Darling and Thomas, 2005). *Lumbricus terrestris* earthworms were found to significantly bioaccumulate both lead acetate and lead carbonate under experimental conditions (Darling and Thomas, 2005). Higher numbers of takahe family groups with and without chicks need to be examined, as well as analysis of food sources to assess if an effect of feeding method exists on potential sources of lead exposure.

Blood lead concentration represents recent exposure to lead, or release of lead from tissue stores such as bone. Bone lead concentrations are more representative of lifetime exposure to lead and may have provided a better indication of lead exposure in takahe, however, the collection of bone samples was precluded due to the invasiveness of the collection procedure on live animals and the destructive potential on valuable skeletons.

The analysis of tissue lead concentrations revealed that birds from the Murchison Mountains had all been exposed to lead. This result was surprising as the Murchison Mountains are considered to be a pristine environment surrounded on three sides by lakes and backed by the Main Divide of the Southern Alps (Maxwell, 2001). The range of the takahe is predominantly within the Takahe Special Area, an area in the Fiordland National Park to which only permitted conservation and research workers are allowed access (Maxwell, 2001). The area is accessible only by helicopter or by boat followed by walking up steep mountain valleys (Maxwell, 2001). The exposure of the birds to lead suggests that there has been lead contamination of even this isolated

environment. The source of the lead is difficult to determine. Shooting from the ground and air has been carried out in the area for deer control for many years, however despite the potential for lead in the bullets and the risk to individuals who may ingest a bullet or bullet fragment, it is unlikely that this form of extensive hunting would heavily contaminate the environment (compared for example with intensive shooting over wetlands with lead shot). The site is far from any point source of lead emissions, however while lead emissions are generally concentrated in industrial areas, small particles have been transported in winds to even the most remote environments including the Arctic and Antarctic (Pattee and Pain, 2003). It is possible there has been post-mortem contamination of the bodies or tissue samples. Most of the specimens had been stored in a freezer in a storage shed for up to 15 years with multiple freeze-thaw cycles during this time. There was no vehicle access to the shed and it is unlikely that contamination with lead would have occurred. Tissue handling during and following collection was carried out with care to prevent inadvertent contamination. Tissue lead analysis was not carried out by the authors, however internal controls at the laboratory suggest contamination had not occurred. Our results therefore suggest that human-induced lead contamination of the environment is extending into formerly pristine areas and exposing free ranging wildlife.

Kidney lead levels from the archived offshore island takahe were found to be significantly higher than those from the Murchison Mountain takahe ($p = 0.035$). This would be expected due to the greater potential for environmental exposure of offshore island takahe to lead. While it is likely that a true difference exists, interpretation of the tissue lead concentrations is difficult due to differences in tissue fixation and storage. Tissues stored in formalin and those frozen for extended periods of time with multiple freeze-thaw cycles will have altered water content. Our results are reported as wet weight, and water loss is likely to have occurred in some tissues, which may have artificially elevated the results reported. Dry weight is considered more accurate due to inconsistent water loss during storage and handling (Adrian and Stevens 1979), however this was not available.

While our study did not indicate a significant clinical problem of lead exposure of takahe, it revealed that a large proportion of both the offshore island and wild population have been exposed. As the takahe are a critically endangered species with poor reproductive success, the effect of exposure to lead on reproductive success may be significant. Further investigation into the effect of lead on offshore island takahe

populations is warranted. This could include seasonal evaluation of blood lead with further comparison of blood lead values in breeding and non-breeding animals with respect to home territory (e.g. association with buildings). Soil testing within territories of birds including and excluding buildings may help establish if the buildings are the source of lead for the takahe. While lead shot exposure was not investigated in this study it may pose a risk for takahe as they commonly ingest grit to aid in the digestion of their food. It is possible that lead pellets could be ingested if present in the environment and should be a consideration when establishing new takahe populations. Lead isotope analysis or organ lead and environmental lead may help in identifying the source of the lead and enable reduction or elimination of exposure. Lead isotope analysis has been used to identify environmental lead sources in endangered species recovery programs such as the red kite (*Milvus milvus)* program in England (Pain et al., 2007).

Any cause of reduced fitness, increased morbidity, or mortality in a threatened or endangered species is of importance, particularly with long-lived, slow breeding species that have restricted population sizes and ranges (Fisher et al., 2006). If lead exposure is found to be a significant problem for offshore island takahe, management measures to ameliorate the effects of lead would be difficult. Despite being intensively managed, handling of the birds occurs infrequently making treatment such as chelation impractical. There is some evidence that dietary calcium, if eaten during the period of lead exposure can reduce the intestinal absorption of lead (Varnai et al., 2001). Many of the takahe are conditioned to eating a pelleted diet in addition to their wild diet and it is possible that additional calcium supplementation could be provided prior to and during the breeding season to help decrease lead absorption. In addition, soil testing and testing of soil dwelling invertebrates such as worms to identify high risk areas could be carried out and if practical, these areas could be fenced to exclude takahe. If stripping of paint or disposal of painted items is to take place on the offshore islands, it would be advisable to follow the precautionary principle and attempt to reduce the amount of lead paint that may be added to the soil. Proper disposal of other risk items such as batteries should be ensured and when performing pest eradication or management, the use of lead ammunition should be considered carefully and caution should be used if potential for environmental contamination exists.

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Chapter 4

Clenched Feet Paralysis and Leg Paresis in Australasian Harriers (*Circus approximans*) in New Zealand.

Abstract

Australasian harriers (*Circus approximans*) are one of three extant native raptor species in New Zealand and are a common species presented to veterinary clinics throughout New Zealand. Nine harriers with clenched feet paralysis and variable degrees of leg paresis were presented to the New Zealand Wildlife Health Centre (NZWHC) at the Massey University Veterinary Teaching Hospital (MUVTH). This study investigated the clinical signs, pathology and response to chelation treatment of clenched feet paralysis and leg paresis in wild harriers in New Zealand. There was a strong association between blood lead concentration and clenched feet paralysis in harriers, although there were features of the response to chelation treatment, electroneurodiagnostics and pathology that were inconsistent with lead poisoning as reported in other birds of prey. Harriers with clenched feet were found to have statistically significantly higher blood lead concentrations than those without clenched feet (t-test; $t = -4.06$, $df = 5$, $P = 0.01$), despite lead exposure appearing to occur at a high level in the local population of wild harriers with 60% of tested birds having elevated blood lead concentrations. Pathological findings were inconsistent with previous reports of lead toxicosis. We conclude that lead is a major factor in the expression of this clinical syndrome of clenched feet paralysis but that other factors not identified in our study are playing a role in the expression of the disease.

Keywords

Australasian Harrier; *Circus approximans*; blood lead; Pb; toxicity; clenched feet.

Introduction

Australasian harriers (*Circus approximans*) are one of three extant native raptor species in New Zealand. They are ubiquitous throughout both the North and South Islands of New Zealand and areas of Australia and prefer open habitat. Harriers are opportunistic feeders and will spend time scavenging roadkill, particularly the introduced brush-tailed possum (*Trichosurus vulpecula*), as well as hunting birds and introduced mammals.

Harriers are a common species presented to veterinary clinics throughout New Zealand. The New Zealand Wildlife Health Centre (NZWHC) at the Massey University Veterinary Teaching Hospital (MUVTH) has been presented with a number of birds showing clenched feet and variable degrees of leg paresis. High blood lead concentrations are recorded in these birds, however chelation therapy has not routinely resulted in resolution of clinical signs. Reported neurological signs of lead poisoning in other raptors include seizures, weakness or depression, regurgitation or other gastrointestinal disease and peripheral neurological signs such as leg paralysis (Locke and Thomas, 1996).

Scavenging and carnivorous birds may ingest lead when feeding on lead affected animals, or animals containing lead shot in their tissues. Acute and chronic poisonings are recognised in wild raptors (Locke and Thomas, 1996; Mateo et al., 2001; Pain et al., 2007). Birds with acute poisoning may present in good condition exhibiting neurological signs such as leg paralysis or seizures, or die without any clinical signs (Dumonceaux and Harrison, 1994; Locke and Thomas, 1996). Chronic poisoning is more insidious and may present as wing droop, reluctance or inability to fly, immunosuppression (indicated by secondary infections), poor growth or weight loss and difficulty hunting, leading to poor condition and death either directly or due to starvation or through misadventure (US Geological Survey, 1999; Pattee and Pain, 2003).

This study aims to investigate the pathophysiology of clenched feet paralysis in harriers using a range of antemortem measures including: clinical and neurological examinations; haematology; plasma biochemistry; evoked and spontaneous electromyography (EMGs); nerve conduction studies; and response to treatment. These

findings were compared with the histopathological appearance of the nerves and muscles of the pelvic limbs.

Materials and Methods

Harrier Admissions, Diagnostics and Treatment

A retrospective review of harriers admitted to the MUVTH between January 2005 and April 2005 was undertaken. Birds that had blood lead concentrations recorded in their file were selected for further examination. Clinical records were examined for information on clinical presentation, blood lead values, other clinical pathological values, response to treatment and post mortem findings. Five birds tested for blood lead concentration with a minimum of clinical presentation recorded were selected for inclusion in the study.

Prospectively, twenty-five harriers presented to the NZWHC from May 2005 until August 2006 underwent a complete physical examination at the time of or soon after admission. Four lead exposed birds with clenched feet and two lead exposed birds without clenched feet underwent a full neurological examination (Appendix 7). Normal birds without exposure to lead were used as controls. Between one and three days after admission birds were anaesthetised with isoflurane in oxygen via face mask and blood was collected from the medial metatarsal, ulnar or jugular veins and placed in 0.4 ml EDTA and lithium heparin microcontainers (Becton Dickenson Vacutainer Systems, Preanalytical Solutions, Franklin Lakes, NJ 07417) for assessment of haematology, plasma biochemistry and blood lead concentrations. Whole body ventrodorsal and lateral radiographs were taken at this time.

Heparinised blood samples were submitted to a commercial laboratory for haematology and plasma biochemistry analysis for the range of metabolites listed in Table 4.2, or in-house biochemistry was performed on 0.09 ml of heparinised blood using a bench top VetScan (Abaxis Inc., Union City, CA, USA) laboratory between 30 minutes and one day following collection. Blood from cases admitted prior to June 2006 (n = 14) was submitted to a commercial laboratory for lead analysis on one millilitre of blood in EDTA anticoagulant using proprietary methods based on colorimetric measurement of trace metals as described in Sandell (1959) . Blood collected after June 2006 (n = 11) was analysed for lead concentration using a portable lead analyser (LeadCare®, ESA Inc, Chelmsford, Massachusetts, 01824, USA). A measured volume

of blood (0.05 ml) was placed in a buffer solution and analysed for lead content immediately after collection. The analyser uses a method known as Anodic Stripping Voltammetry (ASV) as described in Wang (2000), and has a detection range of 0.0mg/L – 0.65 mg/L and an analytical reporting range of 0.014mg/L – 0.65mg/L. (Anonymous, 2005). Blood lead concentrations greater than 0.65 mg/L are expressed as "HI". When this upper limit of the analyser was reached, blood samples were submitted to the commercial laboratory for testing (n = 2).

Four harriers presenting with trauma without neurological signs and with a minimum of normal blood lead concentration (less than 0.1 mg/L), haematology and biochemistry were selected as controls for this section of the study.

Treatment of birds with elevated blood lead concentrations (> 0.1 mg/L) consisted of twice daily chelation with 40-50 mg/kg of calcium disodium ethylenediamine tetraacetate (CaEDTA) (Calcium EDTA Solution 20%, National Veterinary Supplies Ltd, Christchurch) intramuscularly (IM) or intravenously (IV) and a balanced electrolyte solution (75ml/kg IV or per os) for five days. CaEDTA was then discontinued for two days and blood lead, packed cell volume (PCV) estimated white cell counts and uric acid were analysed. Treatment was then reinstituted for a further five days. This cycle of treatment was continued until blood lead levels were less than 0.1 mg/L or the bird was euthanased. Supportive treatment for other presenting problems such as trauma was also provided. A sample admission and treatment sheet is presented in Appendix 6.

Electroneurodiagnostics

For spontaneous and evoked electromyograms (EMGs), all birds were masked with oxygen and increasing percentages of isoflurane in oxygen until they were able to be intubated using size 25-30 Cole endotracheal tubes. The birds were then maintained in a light plane of anaesthesia using inhaled isoflurane in oxygen for the duration of the testing. The birds were kept on towels with heated bags of fluids to help maintain body heat and heart rate was monitored with a stethoscope. Intermittent positive pressure ventilation (IPPV) was used to maintain adequate respiratory rate and depth.

Evoked and spontaneous electromyograms were carried out on four birds with clenched feet following the protocol suggested in Clippinger et al (2000) for pelvic limb nerves. The signal was amplified using a signal amplifier (ISO-Dam, World Precision Instruments, FL) with a gain of 100 and a pass band (low and high filter settings) of 1 –

2 Khz. The signal was digitised with a PowerLab analogue to digital converter (AD Instruments, Dunedin, NZ) and recorded to a personal computer (Toshiba Satellite) using Chart software (AD Instruments, Dunedin, NZ) for spontaneous EMG and Scope software (AD Instruments, Dunedin, NZ) for evoked EMG. Subdermal needle electrodes (Oxford Instruments, Old Woking, Surry UK) were used for stimulating the electrical signal and 26ga concentric needle electrodes (Medelec Old Woking, Surry, UK) were used for recording the signal. No control birds were available for this section of the study.

Spontaneous EMG

Spontaneous EMGs were recorded in the cranial and caudal muscle groups proximal to the stifle (iliotibialis cranialis, flexor cruris medialis and iliofibularis) and the cranial and caudal muscle groups distal to the stifle but proximal to the tarosmetatarus joint (tibialis cranialis and medial and lateral heads of the gastrocnemius) in the right and left legs of four affected birds. Two birds were examined twice during their treatment. A common electrode was placed between the leg being recorded and the heart to decrease noise artefact in the recorded trace. Insertion and resting activities were recorded using Chart software. Electrical activity was examined briefly at the time of recording and then on recorded tracings. The electrical activity stimulated in the muscle when an electrode is inserted (insertion potential) was recorded as a presence or absence and subjectively recorded as normal or reduced.

Evoked EMG

Evoked EMG was used to evaluate nerve conduction in the right and left tibial nerves in three affected birds. Recordings were made in four locations (the cranial and caudal muscles proximal to the stifle, and the cranial and caudal muscle groups distal to the stifle but proximal to the tarsometatarsal joint) along the tibial nerve in the right and left legs. The proximal cathode (stimulus electrode 1) was placed in the greater trochanter area and the distal cathode (stimulus electrode 2) and anode (concentric recording electrode) were placed between 10 and 15mm apart over the area of the tibial nerve of interest. A grounding electrode was placed between the distal stimulating electrode and the recording electrode. The distance between the cathodes and the anodes was measured to enable conduction velocity at different locations along the tibial nerve to be measured if present. The following parameters were used: stimulus delay = 0.5ms;

duration = 0.1ms; amperage = 10V; range = 10V; time base = 10kHz; samples = 256; time = 20ms; and sampling average = 16 sweeps. When no conduction was recorded, the electrodes were reversed to identify if the stimulus artefact would invert.

Conduction studies

Two affected birds and one control bird (blood lead concentration < 0.1 mg/L) were euthanased with an overdose of halothane (Halothane BP VCA, Vetpharm, New Zealand) or pentobarbitone (Pentobarb 300, National Veterinary Supplies, Auckland, New Zealand) and their ischiatic and tibial nerves dissected out. The nerves were bathed in compound sodium lactate (Hartmans) solution (Baxters, Auckland, New Zealand). Using a Grass S48 nerve stimulator (Grass Technologies, West Warwick, RI, USA), voltages were applied to the proximal end of the nerve and recorded distally at the level of the stifle and at the end of the tibial nerve (approximately the level of the hock). As a negative control, lignocaine (MIN-I-JET® Lignocaine Hydrochloride Injection 100mg/10mL, International Medication Systems, Limited, California, USA) was applied to the control nerves and stimulation repeated. The presence or absence of conduction between the hip and stifle and the stifle and the foot was recorded in all three birds.

Pathology

A complete pathological examination was carried out on six lead affected birds with clenched feet and one control bird (blood lead concentration < 0.1 mg/L at the time of presentation). Tissues were preserved in 10% neutral buffered formalin. Blocks of preserved tissues were routinely embedded in paraffin and sections were cut and stained with haematoxylin and eosin. Routine histopathology was carried out on a range of organs such as liver, kidney, spleen, lung, heart, brain and gastrointestinal tract. Representative transverse and longitudinal sections of the cranial and caudal femoral and tibial muscle groups were examined with light microscopy. The ischiatic nerve was dissected from the hip to the hock joint and examined along its length in longitudinal and transverse sections. Representative samples of nerve and muscle sections were stained with luxol fast blue and counter-stained with silver.

Statistical Analysis

Statistical analysis was performed using SigmaStat© for Windows, version 3.5 (SPSS Inc., Chicago, IL 60606, USA). Normally distributed data was compared using a t-test. For the purposes of statistical analysis, blood lead levels below the analytical reporting range of the colorimetric method were reported as 0.1 mg/L and those below or above the analytical reporting range of the LeadCare® analyser were reported as 0.014 mg/L and 0.65 mg/L respectively. Unless specified, means are reported ± standard deviation.

Results

Blood Lead Concentrations

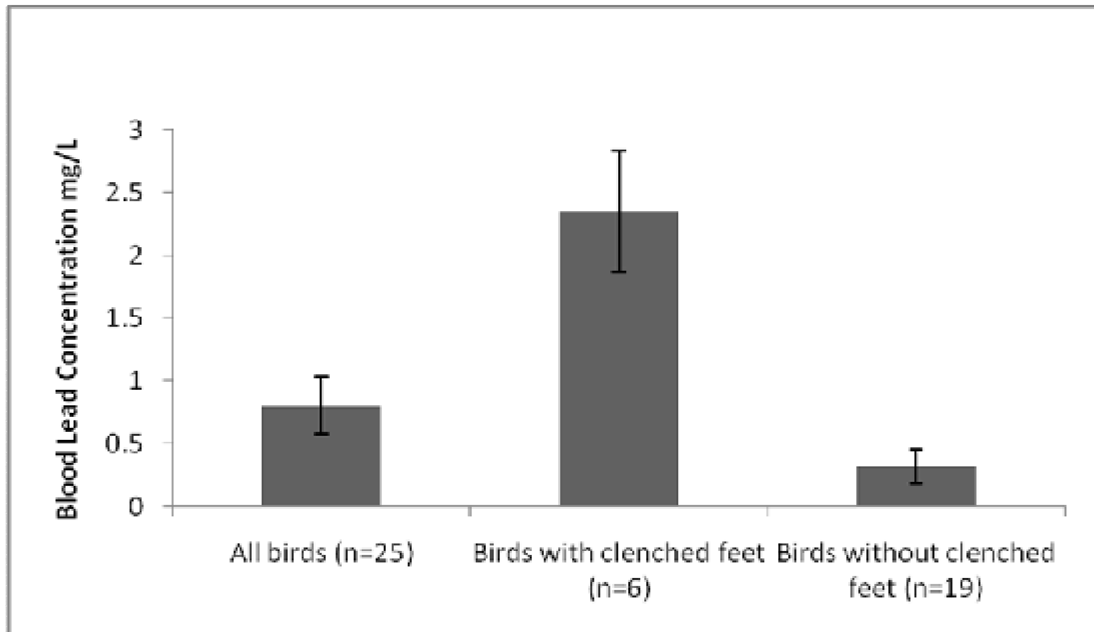
Blood lead concentrations were measured in 30 harriers presenting to the NZWHC between January 2005 and August 2006. Sixty percent (18/30) of these birds recorded blood lead concentrations of 0.1 mg/L or greater. The mean blood lead concentration of 25 of the 30 harriers was 0.806 ± 1.155 mg/L with a range of 0.014 – 3.7 mg/L. Five harriers were excluded from this analysis despite recording elevated lead concentrations due to laboratory or clinician error resulting in an artificially lowered result.

Nine of the 30 birds presented with clenched feet and six of these had admission blood lead concentrations free from error. The mean blood lead concentration of birds presenting with (n = 6) (mean = 2.35 ± 1.179 mg/L; range 0.5 – 3.7 mg/L) and without (n = 19) (mean = 0.318 ± 0.589; range 0.014 – 2.5 mg/L n=19) clenched feet (Figure 4.1) were found to be significantly different (t-test; t = -4.06, df = 5, P = 0.01).

Clinical Presentation and Blood Lead Concentration

For nine birds (30%), the major presenting sign was paralysis of the feet in a clenched position. The flexor tendons were contracted and the toes were knuckled under, with most of the birds presenting with pressure sores and inflammation of the digits. In some birds the toes could be straightened manually, however the birds showed no voluntary movement in the digits and the feet would clench soon after straightening. Most birds showed evidence of pain perception (assessed by pricking the area with a needle) detected by the bird attempting to escape or turning to look at or bite the

Figure 4.1: Mean blood lead concentrations of all 25 harriers, six harriers with clenched feet and 19 harriers without clenched feet admitted to the NZWHC between January 2005 and August 2006. Error bars represent one standard error of the mean.



assessor, or in some birds, with a withdrawal reflex. Seven (78 %) of the birds with clenched feet also had hock paresis and were unable to stand in a normal upright position. Three (33 %) of the nine birds were found to be in poor body condition and one presented with a history of seizures. All nine of these birds had elevated blood lead concentrations. The mean blood lead concentration, 2.35 ± 1.179 mg/L (range = 0.5-3.7 mg/L) was calculated using six harriers due to laboratory error in two cases and sampling error in the third artificially lowering the blood lead concentration

Four birds with elevated blood lead but without signs of clenching were also examined. One of these birds had hock paresis without clenching of the feet. Two of the birds were ataxic and one was presented with a history of seizures. The mean blood lead concentration of the lead affected birds without clenched feet was 1.29 ± 1.02 mg/L (range = 0.65-2.8 mg/L).

A statistically significant difference was not identified between the blood lead concentrations of harriers with clenched feet (n = 6) and those with elevated blood lead but without clenched feet (n = 4), (t-test, t = 2.18, df = 8, P = 0.061).

Neurological Examination

Standardised neurological examinations were performed on four of the nine birds presenting with elevated lead and clenched feet and two of the birds presenting with elevated lead without clenched feet. Birds with clenched feet all had knuckling of digits and no grasping reflex. Three of the four had detectable muscle atrophy of the legs. Birds from both groups displayed abnormalities in leg strength and withdrawal as well as balance loss. Birds from the control group showed no neurological abnormalities. Selected neurological exam results are shown in Table 4.1.

Radiographic Findings

Eight of the nine birds with elevated blood lead and clenched feet were radiographed. Findings included prominent renal shadow in two birds, and a metal fragment was observed in the ventriculus of one bird. Skeletal changes associated with trauma were seen in three birds and three birds had no significant findings recorded. Three birds with elevated blood lead concentrations but without clenched feet were radiographed with two showing evidence of a prominent renal shadow and one bird with skeletal changes associated with trauma. The three control birds radiographed showed evidence of trauma, including one bird that had been shot.

Haematology and Biochemistry

Haematological and biochemical parameters were obtained at the time of admission for six harriers with clenched feet, four lead exposed harriers without clenched feet and four control birds. No significant difference was detected between the PCV of clenched and unclenched lead affected harriers (t-test; $t = -0.666$, $df = 7$, $P = 0.526$: Power $= 0.05$), or the clenched and control harriers (t-test, $t = 1.26$, $df = 4.7$, $P = 0.27$). The mean estimated white cell count (WCC) of the affected birds ($n = 4$) was 17.13 ± 7.6 (range = 10.8 – 27.2). The mean estimated WCC of the lead-exposed non-clenched harriers ($n = 4$) was 8.7 ± 4.1 (range = 3.2 – 12.25).). No significant difference was detected between the two groups (t-test; $t = 1.942$, $df = 6$ $P = 0.1$), however the power of the test was low (0.282). A significant difference was detected between the heterophil count in control harriers compared with clenched-feet harriers (t-test; $t = 2.39$, $df = 10$, $P = 0.038$) with control harriers having significantly higher heterophil counts than affected harriers. Mean white cell counts were not found to be significantly different, however there was

Table 4.1 Selected neurological exam results of harriers presenting with elevated blood lead concentrations.

Test	*Clenched (n = 4)*	*Unclenched (n = 2)*
Abnormal Posture and gait	3/4	2/2
Abnormal Mental Status	1/4	1/2
Abnormal Cranial Nerves	0/4	0/2
Balance loss	2/4	2/2
Proprioceptive Deficits	2/4	1/2
Abnormal Wing Function	1/4	1/2
Leg Function		
• Abnormal Strength and tone	2/4	2/2
• Muscle Atrophy	3/4	0/2
• Loss of Cutaneous pain/touch	1/4	1/2
• Central registration of pain	4/4	1/2
• Abnormal Withdrawal reflex	2/4	2/2
• Abnormal Grasping reflex	4/4	1/2
• Knuckling of digits	4/4	0/2
• Detectable fracture	0/4	0/2
Abnormal Cloacal Function	0/4	0/2

a trend of lower WCCs (t-test; t = 2.22, df = 9.2, P = 0.053) and higher band heterophil counts (t-test; t = -2.75, df = 7.5, P = 0.077) in harriers with clenched feet than controls.

The biochemical parameters are presented in Table 4.2. With the exception of total protein, albumin and potassium analytes, no difference was observed between clenched and unclenched lead exposed harriers. A difference was observed between CK values of clenched and control harriers (t-test; t = -2.254, df = 9.6, P = 0.049). Higher calcium values were observed in the clenched harriers when compared with controls. Statistical significance was not observed (t-test, t = -2.184, df = 9.8, P = 0.054).

Electroneurodiagnostics

Spontaneous EMGs were performed on four affected birds. Examination of the proximal muscle groups did not reveal any abnormal electrical activity. Examination of

the distal muscle groups revealed evidence of possible lower motor neurone disease (LMN) in all four birds examined. Abnormal signs identified included denervation potentials, and reduced or absent insertion potentials.

Evoked EMGs were performed on three affected birds. Conduction was recorded between the hip and the stifle, but not between the stifle and the foot in all three birds. Evoked EMG was performed on the ulnar nerve of one bird and conduction was recorded along its length.

Conduction was present in the proximal ischiatic nerve of two affected and one control bird. There was no conduction in the distal nerves of the two birds with clenched feet, while conduction was present in the control bird. The application of lignocaine to the nerve bath in the control bird resulted in no conduction. Conduction velocities were not calculated. The nerve conduction studies are consistent with an absence of neurological function in the peripheral nerves distal to the stifle in birds with clenched feet.

Treatment Response

Nine birds with clenched feet underwent treatment. Hospitalisation time ranged from four days to 185 days with a mean of 46 days ± 56.6 and a median of 21 days. Five birds showed a reduction in blood lead concentration with chelation treatment (Table 4.3), the remaining four were euthanased before an effect of chelation on blood lead concentration could be demonstrated. A clinical response was observed in one bird who regained foot function over 27 days while blood lead concentration decreased from 3.7mg/L to <0.1mg/L. The bird was sent to a rehabilitation facility however its feet re-clenched during transit and it was readmitted. The bird was euthanased following incomplete resolution after a further five months of treatment.

Three birds with elevated blood lead concentrations without clenched feet underwent treatment. Hospitalisation time ranged from 30-35 days with a mean of 33 days ± 2.65. All three birds showed a reduction in blood lead concentration and a resolution of clinical signs of lead toxicity in response to chelation treatment (Table 4.3). Two of the three birds were sent to rehabilitation for eventual release, one remained in permanent captivity as a retired advocacy bird. The fourth bird was euthanased on presentation due to severe trauma.

Table 4.2 Biochemical and electrolyte values from lead-exposed clenched and unclenched harriers, and control harriers presenting to the New Zealand Wildlife Health Centre between January 2005 and August 2006. Values with significant differences between the unclenched or control harriers and the clenched harriers are highlighted in bold. Values are expressed as mean ± standard deviation (SD) and minimum to maximum range for all samples.. Values reported include creatine kinase (CK), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT) and albumin/globulin ration (A/G ratio). Values are reported in SI units.

Analyte	Clenched harriers (n= 6)	Unclenched lead exposed harriers (n=2)*	Control Harriers (n=3)**
Uric Acid umol/L	514.75 ± 351.36 (156-1023)	665.33 ± 459.50 (151 – 1041)	309.25 ± 146.67 (166 – 503)
CK IU/L 37C	952.38 ± 276.34 (477-1299)	1055 ±407.29 (767 – 1343)	**794 ± 148.96** **(708 – 966)**
AST IU/L 37C	410.25 ± 157.78 (218-738)	339 ± 186.67 (207 – 471)	678.67 ± 520.59 (245 – 1256)
GGT IU/L 37C	3.75 ± 2.96 (2-11)	0.5 ± 0.7 (0 – 1)	2.33 ± 1.53 (1 – 4)
Total Protein g/L	38.13 ± 6.27 (30-46)	**26 ± 1.4** **(25 – 27)**	33 ± 1 (32 – 34)
Albumin g/L	15.13 ± 2.17 (13-19)	**9.5 ± 2.1** **(8 – 11)**	14 ± 1.73 (13 - 16)
Globulins g/L	23 ± 5.21 (16-30)	16.5 ± 3.5 (14 – 19)	19 ± 1 (18 – 20)
A/G ratio	0.69 ± 0.16 (0.5-0.88)	0.6 ± 0.2 (0.42 – 0.8)	0.74 ± 0.13 (0.65 - 0.89)
Calcium mmol/L	2.68 ± 0.18 (2.34-2.89)	2.46 ± 0.08 (2.4 – 2.51)	**2.46 ± 0.07** **(2.39 – 2.52)**
Glucose mmol/L	18.93 ± 1.21 (17.3-20.6)	17.9 ± 0.42 (17.6 – 18.2)	19.4 ± 1.44 (18.2 – 21)
Sodium mmol/L	156.13 ± 3.36 (152-163)	154.5 ± 3.54 (153 – 157)	156.67 ± 3.79 (18.2 – 21)
Potassium mmol/L	2.36 ± 0.48 (1.9-3.2)	**3.6 ± 1.4** **(3.5 – 3.7)**	2.83 ± 0.86 (1.9 – 3.6)
Chloride mmol/L	121.38 ± 5.83 (116-134)	125.5 ± 6.36 (121 – 130)	124 ± 1.73 (122 – 125)

* n = 3 for uric acid analysis.

** n = 4 for uric acid analysis

Table 4.3 Blood lead levels (mg/L) of clenched (n = 9) and unclenched harriers during treatment over a 28 day period. HI represents an elevated blood lead level for which an accurate value was not obtained. C represents clenched, UC represents unclenched, E represents Euthanased.

Harrier	Day1	Day 7	14	21
1 C	0.2*	2	0.5*	0.2
2 C	2.3	E	-	-
3 C	0.3**	E	-	-
4 C	1.5	0.5**	0.3	
5 C	3.7	1.2	0.9	<0.1
6 C	0.5	0.2	E	-
7 C	3	-	0.5	0.1
8 C	HI	E	-	-
9 C	3.1	E	-	-
1 UC	HI *	0.7	0.344	0.143
2 UC	2.82	1.6	0.068	0.026
3 UC	1	0.8	0.2	<0.1

* Lab error
** Blood collected while on chelation

PCV and uric acid levels were monitored weekly during hospitalisation in four affected birds A trend of increasing PCV (Figure 4.2) was observed in these four birds and was found to be significant (one way repeated measures ANOVA: p=0.037) however a pairwise comparison (Holm-Sidak) found that significant differences did not occur between weeks. No differences were observed in blood uric acid concentrations during the treatment period (one-way repeated measures ANOVA: p = 0.681). Raw data is presented in Appendix 8.

Pathological Findings

Histopathological sections of the leg muscles from six affected birds were examined. High numbers of myofibrils showed pathological changes including: centralising nuclei; swollen, hypereosinophilic myocytes; mycoyte necrosis; fatty infiltration and replacement of muscle fibres with fat; regenerating muscle fibres and variation in myocyte size. These pathological changes were found in iliotibialis cranialis, femorotibialis, flexor cruris medialis, iliofibularis, tibialis cranialis and gastrocnemius muscle sections examined from all six birds. Sections from four birds also showed small multi-focal inflammatory foci within the muscles consisting primarily of mononuclear inflammatory cells, however granulocytes were also observed in some areas. One bird had evidence of fibrosis. One harrier with clenched feet had more severe changes within its femorotibialis with inflammation throughout the transverse section of muscle and segmental necrosis of muscle fibres observed in the longitudinal section. Flexor hallucis longus was examined in one affected bird and revealed moderate numbers of cells with centralising nuclei, necrosis and fatty infiltration with variation in cell size and occasional regenerating muscle cells.

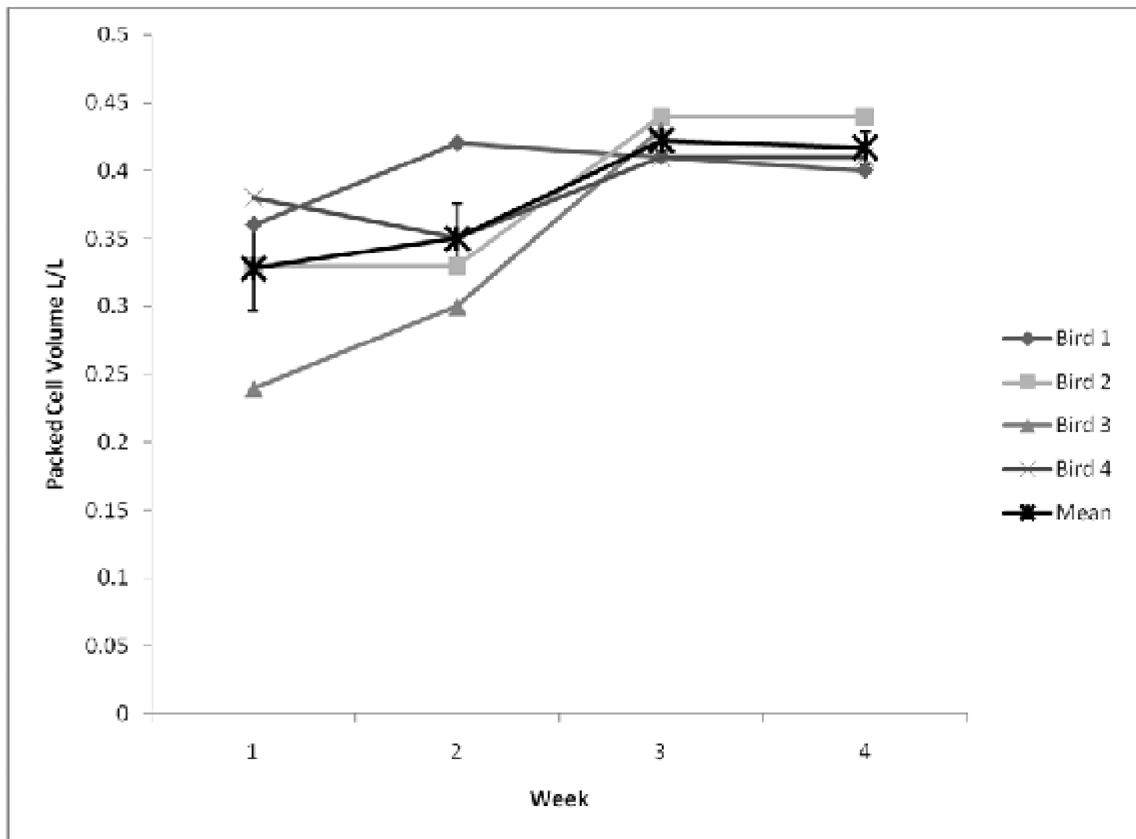
Occasional central nuclei, necrotic cells and regenerating muscle fibres were observed in the muscle sections from the control bird. Sarcocysts were present in almost all sections examined from both affected and control birds but were not associated with any histopathological changes identifiable with light microscopy. No acid-fast intra-nuclear inclusions of the renal tubular cells were observed in six harriers with elevated admission blood lead or in a control harrier. No abnormalities were identified in peripheral nerves of the legs in any birds examined.

Discussion

This study investigated the clinical signs, pathology and response to chelation treatment of clenched feet paralysis and leg paresis in wild harriers in New Zealand. There was a strong association between blood lead concentration and clenched feet paralysis in harriers, although there were features of the response to chelation treatment, electroneurodiagnostics and pathology that were inconsistent with lead poisoning as reported in other birds of prey.

Lead exposure appears to be occurring at a high level in the local population of wild harriers with 60% of tested birds having elevated blood lead concentrations.

Figure 4.2 Increase in mean PCV over four weeks in four harriers with clenched feet* .



*Week 4, n = 3

However, this may represent a biased sample. While the intent was to test every harrier admitted to the hospital for blood lead concentrations, not all birds were tested and birds displaying neurological signs are probably more likely to be tested.

With the exception of the control birds, all of the birds selected for the study into clenched feet paralysis and lead had evidence of lead exposure confirmed by analysis of blood lead concentration from blood collected at, or soon after admission. Not all birds with elevated blood lead concentrations exhibited clenched feet paralysis. It is possible that the birds with clenched feet had chronic toxicity, with a greater exposure to lead or that there is another cause of the clenched feet syndrome. Radiographs taken of each bird rule out skeletal damage as a cause of the peripheral nerve signs in the legs, although spinal trauma without vertebral fractures could not be excluded based on these findings.

A neurological examination was performed to identify differences between neurological and non-neurological conditions and to attempt to assess the degree of

dysfunction. Neurological examinations are difficult to perform on avian patients, particularly raptors and results can vary between clinicians. Birds, particularly wild birds, can have reduced responsiveness during examination making them difficult to assess for neurological function or dysfunction. Postural reactions have not been well studied in birds, however as suggested by Jones and Oroz (1996), mammalian postural tests can be modified for use in birds. A standardised neurological examination (Appendix 7) was developed for examining the harriers during the course of the study in an attempt to reduce variability between clinicians and assist in distinguishing normal from abnormal based on information on avian neurological examination from Jones and Oroz (1996). The neurological exam findings show that detectable neurological deficits were primarily confined to the legs in the birds with clenched feet. A limitation of the neurological exam is the differentiation between nervous system and musculoskeletal disease in the legs. Radiography assisted in ruling out skeletal disease, however differentiating between nervous system dysfunction and muscular disease was difficult.

Haematology and biochemistry results were found to be similar between the two groups of lead exposed birds and between birds with clenched feet and controls. Total protein and albumin were found to be different between the two groups, however assessment of avian proteins by standard laboratory methods is not considered ideal and interpretation with protein electrophoresis may have provided more accurate results (Hochleithner, 1994). No differences were seen in admission PCV between lead affected harriers and between clenched feet and control harriers, however an increase in PCV over four weeks was observed in clenched feet harriers undergoing treatment. This suggests that the PCV of affected harriers was depressed (although not significantly), or that all harriers admitted to the NZWHC have decreased PCVs. Haematology is not always useful in the early diagnosis of lead exposure in an individual, however slight reductions in PCV and/or haemoglobin concentration may be seen when looking at trends in a population (Lauwerys and Hoet, 2001). Reduction in haemoglobin concentrations due to the effects of lead on aminolevulinic acid dehydatase (ALAD) and haeme synthetase can occur following lead exposure leading to anaemia (Pattee and Pain, 2003). Anaemia may be worsened by increased red blood cell fragility, decreased life-span and decreased production (Pattee and Pain, 2003). Harriers with clenched feet were found to have significantly lower absolute heterophils than control birds and a trend towards lower white cell counts and higher band heterophil counts (although these were not considered significant). Lead is known to cause immune system effects

increasing the susceptibility of an animal to disease (Pattee and Pain, 2003). Lower mean circulating white cell counts were found in male mallards exposed to lead in the field and experimentally .(Rocke and Samuel, 1991)

Treatment of lead poisoning in all animals relies on chelation and fluid therapy (Dumonceaux and Harrison, 1994; Locke and Thomas, 1996; Nelson and Couto, 1998) Both lead and calcium EDTA are nephrotoxic. Measures of renal function in birds are limited to uric acid (UA) due to their uricotelic nitrogen metabolism. Blood UA concentration was measured weekly during treatment with CaEDTA to assess renal function. However, avian kidneys have a large functional reserve and significant damage is required before pathological hyperuricaemia occurs. UA can increase significantly following a high protein meal particularly in carnivorous birds. To avoid this, fasting samples were taken. No differences were seen in blood UA concentrations during the treatment period. These findings suggest that prolonged use of CaEDTA using a five day on, two day off treatment protocol is unlikely to result in sufficient renal damage to cause an elevation in UA in well hydrated avian patients. Treatment of these harriers with Ca EDTA was successful in producing a decline in the blood lead concentration over time as detected by serial blood lead measurements.

In mammals, electrodiagnostic testing is used for localisation of neuromuscular lesions and helping to determine prognosis (Bennett, 1994). While less frequently used in birds, electrodiagnostics have been used to determine the prognosis for continuing treatment in wild birds with prolonged rehabilitation (Shell et al., 1993; Holland and Jennings, 1997). In this study, electrodiagnostic testing of the legs suggested an absence of neurological function in the peripheral nerves distal to the stifle. Spontaneous and evoked electromyography (SEMG and EEMG) enables assessment of the nerve and muscle function in live patients. SEMG examines the health of the muscle cells and the integrity of the muscle unit including muscle and nerves, while EEMG examines the ability of a compound action potential to be propagated by a peripheral nerve in response to electrical stimulation (Clippinger et al., 2000). Conduction velocity of freshly dissected nerves enables the health of the nerve along its length to be assessed while measuring the speed of the impulse (Klappenbach, 1995). Reductions in insertion potential activity can be suggestive of reduced muscle responsiveness such as with atrophied muscle (Sims, 1996). While this was observed in some birds, lack of normal recordings for the length of insertion potentials in this species make it difficult to draw definitive conclusions. SEMG indicated that there was possible abnormal electrical

activity in the distal muscle groups. Denervation or fibrillation potentials, observed as biphasic or triphasic potentials are caused by the firing of single muscle fibres in denervated muscle and may be caused by neurogenic or myopathic disorders (Sims, 1996). The frequency of denervation potentials decreases over time as the muscle further atrophies (Sims, 1996).

In retrospect, audio-analysis and more in-depth interpretation should have been carried out at the time of recording rather than saving traces for future analysis. A combination of a recorded tracing and auditory analysis in addition to examination of muscular response to stimulation or probe insertion provides the most accurate assessment of any pathological changes (Bennett, 1994).

Evoked EMG involves repeated stimulations of a nerve and motor units to achieve a recording of a compound muscle action potential (CMAP). This was done to rule out or detect diseases such as those of the neuromuscular junction (seen as a decreasing amplitude of the CMAP), or demyelination (seen as lower amplitude CMAP) however as normal values were not established, subtle abnormalities were therefore unable to be detected. Abnormalities in conduction were observed as complete loss of the CMAP when recording was attempted between the stifle and the foot. This was also observed when the nerves were dissected out and conduction was evaluated. EEMG was advantageous over conduction studies as it enabled this to be determined in an anaesthetised live bird rather than a nerve dissected from a euthanased bird.

Pathological examination of the nerves and muscles of the legs of six affected birds failed to establish an aetiology for the condition. Surprisingly, there was a complete lack of observable histological change present in peripheral nerves examined by light microscopy. This was contrary to the presenting signs, the lack of detectable conduction in some nerves and elevated blood lead concentrations. Lead is known to cause segmental demyelination and axonal degeneration in many species (Hunter and Wobeser, 1980; Verity, 1997; Dart et al., 2004). Demyelination is thought to be caused by toxicity to Schwann cells (Verity, 1997) which may progress to axonal degeneration. Demyelination results in generalised weakness and symptoms such as foot drop and hand drop in humans (Anderson et al., 1996), and leg and wing paresis or paralysis in birds. In cases of chronic lead exposure, different stages of demyelination and remyelination may be seen histopathologically (Feldman, 1977). In lead poisoned mallards, Hunter and Wobeser (1980) found swelling and fragmentation of myelin nerve sheaths occurring in the vagus, brachial and sciatic nerves. In some cases clinical

disease in peripheral nerves may precede disease in other tissues (Hunter and Wobeser, 1980).

While no evidence of lead induced neuropathology was observed in the sections examined, moderate to severe muscle pathology was observed in all the affected birds This was particularly evident in the muscles distal to the stifle, although one bird was found to have severe changes in its femorotibialis muscle. None of the muscular histological changes identified are specific to an aetiology, however muscle fibre degeneration and disintegration with no inflammatory response has been observed in mallards dosed with lead (Clemens et al., 1975). It is possible that prolonged recumbency may have contributed to the muscle changes observed. Prolonged chelation therapy and reduced blood lead concentrations in these harriers may have reduced the ability to detect acute microscopic changes in the nerves, however, chronic changes such as demyelination and remyelination would have been expected.

Lead is known to cause nephrotoxicity in other species via acute tubular necrosis from lead induced cell membrane or mitochondrial damage. (e.g. Confer and Panciera, 1995). Acid-fast lead-protein complexes may form within the nucleus of tubular epithelial cells and demonstration of these inclusions in histological sections is considered consistent with lead toxicosis (Confer and Panciera, 1995). Acid-fast intra-nuclear inclusions of the renal tubular cells were not observed in six harriers with elevated admission blood lead and clenched feet or in a control harrier in this study. However the lead exposed birds that had renal histopathology performed had undergone chelation therapy and had been hospitalised without access to lead for prolonged periods prior to necropsy. If affected, the renal-tubular cells may have sloughed and been replaced during the period of chelation without further lead exposure (Gwaltney-Brant, 2002; Dart et al., 2004). Bone-lead analysis may have been a more useful analysis of body lead in these birds as over half of absorbed lead is eventually deposited into the bones and the half life of lead in bone is considerably longer than in soft tissues (Summers et al., 1995; Lauwerys and Hoet, 2001).

In this study, birds with clenched feet paralysis had clinical signs and electroneurodiagnostic findings most consistent with a peripheral neuropathy and/or myopathy. The birds all had elevated blood lead concentrations and mild anaemia that was responsive to chelation therapy. However, many birds failed to respond clinically to chelation therapy and one bird that showed an apparent response to treatment

demostrated a clinical relapse with transport stress. With no evidence of neuropathology and EMG and CV testing indicating a problem with nerves distal to the stifle, the clinical syndrome is most suggestive of a functional distal neuropathy.

Delayed organophosphate polyneuropathy (DOPN) is a potential differential diagnosis or contributing cause of the clenched feet syndrome. DOPN is a condition affecting both the central and peripheral nervous system occurring one to three weeks after organophosphate (OP) exposure (Bennett, 1994). However, distal axonal degeneration followed by secondary demyelination is usually seen which is not consistent with our findings. Blood cholinesterase levels were measured in some birds at the beginning of the study (Appendix 8) however blood cholinesterase indicates recent exposure and does not give an indication of severity and as DOPN does not appear to result from inhibition of acetylcholine this investigation was discontinued. Future studies should examine either brain cholinesterase or concentrations of OPs in fat to examine the possible role of these toxins in the clenched feet syndrome.

Vitamin B2 deficiency has been reported as causing a curled-toe paresis in chickens and budgerigars with similar clinical signs to the harriers in our study, however pathological changes such as a demyelinating neuritis and oedema in the nerves were observed compared to the lack of histological change in the nervous tissues examined in our study (Bennett, 1994). Further, a dietary deficiency of this nature is unlikely in a carnivore consuming whole carcasses.

While lead may not have been the primary cause of the clinical presentation of the birds in this study, high levels of lead, consistent with those known to cause clinical toxicity, were found in these birds. Harriers are known to eat live prey, but are also very commonly seen scavenging roadkill and dead animals on farmland. Birds suffering from chronic lead exposure may be more likely to scavenge from roadsides resulting in an increased incidence of road trauma. These animals may be presented to veterinarians as primary trauma cases resulting in under-diagnosis of lead poisoning. Conversely, harriers suffering from clenched feet paralysis may scavenge from roadsides resulting in increased lead exposure.

Scavenging and carnivorous birds may ingest lead when feeding on lead affected animals, or animals containing lead shot in their tissues. While New Zealand has recently banned the use of lead shot within 200m of waterways (Fish and Game New Zealand, 2007), its use is still present in other shooting situations such as clay target,

rabbit and possum shooting. Studies overseas show that despite the banning of lead shot there is still considerable risk of poisoning from previous contamination of the environment (Hoffman et al., 2002; Stevenson et al., 2005). New Zealand still has a very large proportion of its land dedicated to agriculture and this open habitat is favoured by harriers. Possums and rabbits shot as pests may be left out for animals to scavenge and animals that have been shot but not killed may go on to be killed on the road and subsequently scavenged. One bird in the lead only group in this study was in care at the time of intoxication and was being fed roadkill possum (Dawn Morton personal communication April 12, 2005).

These birds are likely to be good ecological indicators of lead in the environment in New Zealand and highlight the potential threat to more endangered species such as New Zealand falcon and waterfowl such as blue ducks and brown teal.

All the harriers in this study were presented at an advanced stage of the disease. Earlier presentation would certainly be advantageous for diagnostic and treatment purposes, however capturing birds that are strong enough to fly and are still able to scavenge food from roadsides is particularly difficult. Post-mortem examination of birds immediately following presentation may assist in ruling out prolonged hospitalisation and recumbency as a cause of the muscle changes, and allow further examination of the effects of lead on the tissues. Bone lead analysis in addition to blood and tissue lead analysis would give a better indication of the chronicity of the lead exposure. Electron-microscopy of the affected nerves may also be useful to look for changes at cytoplasmic level. Electromyography may be useful in further examining the function of the nerves and muscles of the legs, however a better understanding of normal neurophysiology in harriers, modification of existing techniques and using audio as well as visual analysis of traces is required.

While the stated aims of identifying the causal factors leading to clenched feet paralysis and leg paresis in wild harriers in New Zealand and developing a diagnostic and treatment protocol for these birds were not achieved during the study period, this investigation contributed significantly to the knowledge of clenched feet paralysis and leg paresis in harriers. Further investigation is required to continue to work towards identifying the cause of this problem.

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Chapter 5

General Discussion

This study has demonstrated that a diverse range of New Zealand's wildlife in varying habitats are exposed to significant levels of lead from their environment. Kea, inquisitive and destructive mountain parrots, ingest lead from a large variety of sources and are likely to be exposed from hatching to adulthood. This exposure, as well as causing significant mortality, may have an effect on their ability to learn behaviours critical to survival in alpine regions. Takahe, critically endangered flightless rails, were found to have detectable blood lead concentrations in highly modified island environments and lead in their tissues in regions as remote as the inaccessible and supposedly 'pristine' Murchison Mountains. There is potential for lead to have a detrimental sub-clinical effect on breeding success and survival of these birds despite the documented blood lead concentrations being comparatively low. Large numbers of Australasian harriers presenting to the New Zealand Wildlife Health Centre (NWHC) were found to have evidence of lead exposure, which showed a strong association with a clinical syndrome of clenched feet paralysis, although direct causality was not conclusively demonstrated.

The species studied in this thesis are sensitive eco-indicators of the extent of lead contamination of the environment in New Zealand. This study covered habitats as diverse as a remote but heavily visited mountain village (kea), rural farming areas (harriers) off- shore islands and remote alpine wilderness (takahe) and all these diverse habitats contain sufficient lead in their environments to cause exposure that is likely to have serious biological effects.

With the exception of the harriers, the majority of birds examined in these studies were not exhibiting overt clinical signs of acute lead intoxication. To make a definitive diagnosis of lead poisoning, elevated tissue lead concentrations need to be supported by clinical and/or pathological findings (Franson, 1996; Pain, 1996; Hoffman et al., 2002), however the definition of toxic tissue levels consistent with lead poisoning varies between and within species, and lead intoxication in many species has not been studied. This is not surprising given that there is a large range of clinical and sub-clinical effects of lead exposure influenced by variables such as chronicity and amount of exposure, species and life stage.

Thresholds for toxicity are difficult to define as the effects of lead on living organisms vary depending on the chemical and physical characteristics of the lead, route of exposure, diet, other elements ingested, and species and life stage of the

individual (Pattee and Pain, 2003). The effect of lead varies considerably between species and even between individuals within species and is dependent on the chemical properties of lead as well as biological and environmental factors (Pattee and Pain, 2003). As a result, reference ranges for lead indicating "background", subclinical or toxic exposure should be used with caution.

In veterinary medicine, as has happened in human medicine, we need to shift our thinking from focussing on clinically symptomatic animals affected by high doses of lead to considering the subclinical effects of low doses of lead (Needleman, 2004). Gilbert and Weiss (2006) suggest that blood lead levels greater than 0.02mg/L in humans should be regarded as elevated due to the effects of even very low levels of lead on biological processes, particularly the nervous system. As studies of chronic low level lead exposure reveal more about the effects of even small amounts of lead on behaviour, the immune system and reproduction, we would be well advised to consider the precautionary principle and reduce the lead exposure of not just our endangered species, but all species including ourselves. As Lin-Fu (who helped write the American Surgeon Generals statement) commented "*one does not wait to remove known poisons until symptoms occur, but rather removes them as soon as ingestion occurs.*" (Berney, 1996).

The success of a species is measured by survival and successful reproduction and is an increasing challenge for wild animals, especially in today's highly modified environments, or for restricted or highly fragmented populations. The impact of toxins such as lead on normal body function may compromise an animal's ability to reproduce, raise young or to respond appropriately to other factors such as disease or predation. Chronic subclinical toxicities may have an even greater effect on populations than acute poisonings (Friend and Thomas, 1990). Large robust populations of animals may be able to sustain low losses in the form of deaths or losses from the breeding population; however losses can be very significant for small, endangered populations (Friend and Thomas, 1990). Further, any cause of reduced fitness, increased morbidity or mortality in a threatened or endangered species is of importance, particularly with long-lived, slow breeding species that have restricted population sizes and ranges (Fisher et al., 2006).

International examples of species continuing to be threatened in part due to lead intoxication include Stellars sea eagle, Spanish imperial eagle, whooping cranes, Californian condors and white rumped vultures (Fisher et al., 2006). Lead in the

environment may be a concern in reintroduction programs, particularly in areas where waterfowl hunting has occurred, but also in other areas where shooting has taken place (Fisher et al., 2006) Other sources of environmental lead such as old buildings and lead in the soil due to paint or other prior land use may also be a concern.

Kea will continue to visit areas of human habitation such as Mt Cook/ Aoraki village to scavenge food and probably out of curiosity. Currently, the greatest threat to kea in these areas is toxins and garbage ingestion. The removal of construction materials containing lead from all buildings in the area including mountain huts is essential to reduce the impact of lead on the kea. The development of building codes requiring that materials containing lead be excluded from structures to be built in the range of the kea should be developed to ensure exposure does not continue. Less toxic building materials are available and should be utilised in all construction. Garbage, including toxic items such as electrical equipment should be stored carefully to avoid access by kea, remembering that kea are highly intelligent and very quick to learn how to open bins.

A more extensive investigation into the extent of lead exposure in the kea population including testing at other locations frequented by kea should be carried out to assess if other populations are also affected. In addition, bone lead and egg-shell lead concentrations will assist in looking at chronicity, lifetime exposure and risk to young birds. Following removal of sources of lead from the keas' environment, a follow-up survey should be carried out to assess if blood lead concentrations subsequently reduce. Collaboration with behavioural researchers will be important to examine the effect of lead on the behaviours and behavioural development of wild kea. As all the kea in this study were individually identified, behavioural observations and correlation with lead exposure may enable identification of behavioural changes. Education of residents and visitors alerting them to the risks of toxins to kea may be valuable, as well as the collection of observation data from residents and visitors to establish locations of kea sightings and evidence of any unusual behaviour that may be related to lead exposure.

Takahe experience low breeding success and low juvenile survival (Jamieson and Ryan, 1999) on islands. The contribution of lead (and other potential toxins) needs to be examined further. Due to the increased likelihood of lead being ingested during chick raising, studies could focus on breeding pairs looking at serial blood lead

levels, home range and lead content of soil and earthworms. As soil is the most likely source for lead ingestion in takahe, consideration should be given to excluding birds from soils potentially containing lead, particularly those around old buildings. Bone lead concentrations give a much greater indication of lifelong lead exposure than soft tissues so examination of bone lead in birds from all locations would be useful in determining the extent and possibly severity of the problem.

Further investigation of clenched feet paralysis in harriers is required. Pathological examination including analysis of tissue lead concentrations prior to the initiation of chelation therapy should be carried out. Discussion with wildlife carers and veterinary clinics seeing harriers in other parts of the country may be useful to establish if the condition is restricted to the area near the NZWHC. Examination of cases over several years will enable identification of seasonal and/or age and sex variability. The extent of lead exposure in the New Zealand Harrier population could be examined through testing of all harriers admitted to veterinary clinics.

New Zealand is distinctive in that it has no endemic terrestrial mammals other than microchiroptera and is home to a large number of unique avifauna, birds that have evolved throughout centuries of isolation to fill all ecological niches, many of which are filled by mammals elsewhere. Sadly, many of these birds are already extinct or threatened. Lead is one of the most toxic metals known to man, and the current environmental distribution is due primarily to humans. While not all exposure is avoidable, exposure reduction can be achieved using methods such as removal of lead building materials within kea habitat and exclusion of takahe from potentially lead contaminated soils. Other endangered species such as whio (blue duck) or karearea (NZ falcon) also live in habitats or have feeding habits that may put them at risk of lead exposure from environmental contamination, most likely due to ingestion of lead shot. The move to phase out the use of lead shot for hunting in New Zealand will go a long way towards reducing environmental contamination and subsequently reducing the risk for New Zealand's wildlife.

In the species studied in this thesis, the source of lead has been speculated, however radio-isotope testing of the lead found in the body and in the environment will assist in more accurately locating the source(s). This method of identifying the source of lead has been utilised in studies including the recovery program for the red

kite (*Milvus milvus)* in England (Pain et al., 2007). Once identified, the feasibility of ameliorating the environmental contamination can be assessed.

I began this thesis with a quote written 222 years ago by Benjamin Franklin "*You will see by it that the opinion of this mischievous effect from lead is at least above 60 years old; and you will observe with concern how long a useful truth may be known and exist before it is generally received and practis'd on*". This thesis demonstrates that the New Zealand ecosystem is widely contaminated with lead, and despite lead being a known toxin for many centuries, its full effects on biological systems are still largely unknown.

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Chapter 6

Appendices

Appendix 1: Common and Scientific Names Used in Text

Common Name	Scientific name
AVIAN SPECIES	
American woodcocks	*Scolopax minor*
American black ducks	*Anas rubripes*
Bald eagle	*Haliaeetus leucocephalus*
Californian condors	*Gympnogyps californianus*
Canada goose	*Branta canadensis*
Cape pigeon	*Daption capense*
Common Terns	*Sterna hirundo*
Golden eagles	*Aquila chyrsaetos*
Gulls	*Larus spp.*
Herring gull	*Larus argentatus*
Kea	*Nestor notabilis*
Laysan albatross	*Diomedea ummutablis*
Mallards	*Anas platyrhyncos*
Marbled teal	*Marmaronetta angustirostris*
Marsh harriers	*Circus aeruginosus*
Mute Swans	*Cygnus olor*
Red-tailed hawks	*Buteo jamaicensis*
Ring-necked ducks	*Aythya collaris*
Pacific common eider	*Somateria mollisima v-nigrum*
South Island pied oyster catchers	*Haematopus ostralegus finschi*
Spectacled eiders	*Somateria fischeri*
Takahe	*Porphyrio hochstetteri*
Teal	*Anas crecca*
Turkey vultures	*Cathartes aura*
Western bluebirds	*Sialia Mexicana*
White-headed duck	*Oxyura leucocephala*
MAMMALS	
Cat	*Felis catus*
Cattle	*Bos Taurus*
Dog	*Canis familiaris*
Mouse	*Mus musculus*
Mustelids	*Mustela spp.*
Common brushtailed possum	*Trichosurus vulpecula*
Red Dear	*Cervus elaphus*
Rat	*Rattus rattus*
Sheep	*Ovis ovis*
Shrew	*Myosorex varius*
Stoat	*Mustela ermine*
ANNELIDS	
Earthworms	*Lumbricus terrestris*

Appendix 2: Lead Determination Methodology

Blood Lead Determination Using Anodic Stripping Voltammetry

In anodic stripping voltammetry (ASV), metals are pre-concentrated using electrodeposition onto an electrode (Wang, 2000; Bond, 2002). Following a rest stage to allow equilibration, the metal is measured by stripping it off back into solution (Wang, 2000; Bond, 2002). The LeadCare portable lead analyser (Leadcare®, ESA Inc, Chelmsford, Massachusetts, 01824, USA) uses ASV and enables field or "patient side" analysis of blood lead, providing results in approximately 3 minutes. The analyser has a detection range of 0.0mg/l -0.65mg/l and an analytical reporting range of 0.014mg/l – 0.65mg/l (Anonymous, 2005). Levels great than 0.65mg/l are expressed as "HI".

Method

Following blood collection into lithium heparin or EDTA anticoagulants, 50 µL of blood was subjected to acid digestion in 2 ml of HCL solution, inverted several times and allowed to stand for a minimum of 60 seconds. Samples were either analysed immediately or refrigerated and analysed within the time frame recommended by the manufacturer. 30-50µL of the prepared samples was deposited onto an electrode strip that is then inserted into the analyser. Within three minutes a result appears on the screen in micrograms/decilitre (µg/dL). Where the upper limit of the analyser was reached, a 1:10 dilution with saline was used to give a quantitative measure.

Tissue Lead Determination Using Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) is a type of atomic spectrometry and is a much newer technique than the colourmetric methods described below, having been developed in the later half of the 20th century (Dean, 2005). For analysis of clinical samples, ICP-MS is currently the most commonly used technique throughout the world (Vonderheide et al., 2006). The molecular weight of elements or compounds can be measured using mass spectrometry (MS) (Dean, 2005). MS is able to separate elements based on their mass/charge ratio which enables stable isotope

detection (Dean, 2005). ICP-MS has low detection limits and high sensitivity and specificity, and is able to rapidly analyse samples. A nebulisation process is used to convert a sample (usually a liquid) into an aerosol which is then transported by an argon gas stream to the plasma where the element of interest is atomised and ionised. The ions are then extracted, separated and measured by a mass spectrometer (Taylor, 2001). For further details about ICP-MS, the reader is referred to Hill (2006), Dean (2005) and Taylor 2001.

Method

Fresh-frozen and formalin fixed liver and kidney samples submitted to the Massey University Nutrition Laboratory were analysed for lead content using proprietary techniques based on inductively coupled plasma mass spectrometry (ICP-MS) An example of the sample preparation process from Dean (2005) are given below. These methods may differ from those actually used in the processing of the samples in this study.

Samples of tissue were homogenised and then subjected to acid digest using nitric and/or perchloric acid. The prepared samples were introduced to the chamber using nebulisation/spray chamber arrangement. The ICP component turns the sample into ions which are then extracted by the MS according to their weight/charge ratio. The MS also measures the quantity of ions at each mass.

Tissue Lead Determination Using Colourimetric Metal Analysis

The basis of colourimetric analysis is to measure the differential absorption of light of different wavelengths in a coloured solution. The element to be measured is converted in a solution or suspension that is strongly coloured and the percentage of transmitted light is measured and plotted against the wavelength to produce a transmission curve or the percentage of absorbed light is measured and plotted against the wavelength. The colour of a sample solution is compared to the colour of a standard solution of known quantity and white light is passed through the samples and the transmission of light through the sample solution is compared to the standards. Extensive separations and extractions are required to transfer the lead to a final solution which is then measured. These processes are laborious and a source of error due to human error, loss of sample or interference from other elements. To ensure an

accurate result, these errors must be reduced through careful processing. Colourmetric analysis of lead has a reported sensitivity of 1ppm (Sandell, 1959)

Method

Fresh and fresh-frozen liver and kidney samples submitted to a commercial laboratory were processed using proprietary methods based on colourmetric analysis of trace metals (Sandell, 1959). Actual methods may differ slightly from those reported below but cannot be presented due to reasons of commercial sensitivity.

Separation of lead was performed using dithizone extraction. The method recommended for biological materials (where there may on only be small amounts of lead) in Sandell (1959) is to combine a measured amount of the sample with an alkaline citrate solution and dithizone to create lead dithizone which is then extracted with carbon tetrachloride. This extract is then shaken with a dilute acid to decompose the lead dithizone, transferring ionic lead into the aqueous phase where it can be determined.

Sample preparation

Acid-digest is performed on each sample using an acid source such as perchloric and/or nitric acid. 10ml of concentrated nitric acid is added to 4-5gm of the sample to be tested. This is heated gently until the initial vigorous action has subsided, then heated to boiling until the contents have nearly boiled dry. 10ml of 1:1 nitric acid and 10 ml of 70% perchloric acid is added and the sample is heated gently until boiling. The sample should be boiled until all organic material is removed from the sides of the beaker and the solution in almost colourless. The solution is evaporated and 5ml of 1:1 hydrochloric acid and 10ml distilled water is added. This is warmed until all soluble material is in solution. Any insoluble material should be filtered off and washed with hot dilute hydrochloric acid. The combined filtrate and washings are cooled to room temperature and 2 ml of ammonium citrate solution, 10ml of 10% sodium hexametaphosphate and a few drops of thymol blue indicator are added. Ammonia is then added to bring to a pH of 9-9.5 (this corresponds to a blue-green colour).Once cool, 1ml of 10% potassium cyanide and 1 ml of 20% hydroxylamine hydrochloride solution are added. The solution, which should be 40-50ml, is then transferred to a separatory funnel. A control sample (known as a blank) should be run in parallel.

Dithizone extraction:

5ml of dithizone solution (dithizone combined with carbon tetrachloride) is added to the prepared sample and shaken for 30seconds. Once the phases have separated, the carbon tetrachloride phase should be transferred into another separatory funnel. This is repeated with a further 5ml of dithizone. No red colour should be visible. 10ml of water with a drop of 1:1 ammonia is added to the combined extracts and shaken. The carbon tetrachloride phase is drawn off into another separatory funnel. The aqueous phase is shaken with approximately a millilitre of carbon tetrachloride and this is added to the washed carbon tetrachloride extract.

To remove the lead from the carbon tetrachloride extract, the sample is shaken with 5ml of 1:100 nitric acid for 30seconds. The phases are separated and the procedure repeated. The acid fractions are combined in a separatory funnel and rinsed with 1-2ml of water. Any remaining droplets of dithizone can be removed by shaking with a small amount of carbon tetrachloride and drawing them off. If carbon tetrachloride remains on the surface of the solution, the solution should be left to stand unstoppered until it has evaporated. The stem of the funnel is dried with filter paper.

Lead determination

10ml of ammonia-cyanide-sulphite solution and 10ml of 0.001% dithizone solution are added to the sample solution and shaken well for 30 seconds. The phases should be left to separate. A few drops of the clear carbon tetrachloride solution should be allowed to run out of the stem of the funnel, and then some solution can be run into a 1 or 2cm cell. The absorbance of carbon tetrachloride in the reference cell at 520 nm should be obtained immediately and exposure of the sample to strong light should be avoided. A standard curve can be established by taking known amounts of lead and diluting it in 1:100 nitric acid to produce a volume of 10ml. 10ml of ammonia-cyanide-sulphite solution and 10ml of 0.001% dithizone solution are added and the sample shaken well. With sample lead concentrations of up to 3.5ppm in the carbon tetrachloride phase, absorbance at 520 nm varies linearly. The quantity of lead in the sample can be calculated by subtracting the absorbance of lead in the control sample.

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Appendix 3: Raw Kea Data

Table A3.1 Kea Details and Blood Lead Levels

Date	Number	Right band	Left band	Age	Sex	Condition /5	Blood Pb mg/L
19-Apr-2006	1	re/bl	L-14699	Juvenile	Male	3	0.213
19-Apr-2006	2	gr/bl	L-14700	Juvenile	Male	2.5	0.148
19-Apr-2006	3	ye/gr	L-14698	Juvenile	Male	3	0.237
20-Apr-2006	4	re/ye	L-14696	Juvenile	Male	2.5	0.267
20-Apr-2006	5	ye/ye	L-14695	Juvenile	Male	2.5	0.216
20-Apr-2006	6	L-4694	or/re	Juvenile	Male	3	0.247
21-Apr-2006	7	re/gr	L-14693	Juvenile	Male	3	0.292
21-Apr-2006	8	ye/or	L-14692	Juvenile	Male	2.5	0.69
21-Apr-2006	9	bl/ye	L-14660	Juvenile	Male	3	0.135
21-Apr-2006	10	ye/wh	L-14659	Juvenile	Male	2.5	0.087
23-Apr-2006	11	gr/or	L-14658	Juvenile	Male	3	0.328
24-Apr-2006	12	wh/bl	L-14657	Juvenile	Male	3	0.162
2-Dec-2006	13	R37866	Bl/or	Subadult	Female	3	0.23
3-Dec-2006	14	L20342	Bl/green	Adult	Male	2.5	0.028
4-Dec-2006	15	bl/wh	L-14656	Subadult	Male	2.5	0.293
21-Feb-2007	16	Bl/bk	L-14690	Fledgling	Female		0.297
21-Feb-2007	17	O/Y	L-14651	Fledgling	Male		0.285
22-Feb-2007	18	bk/or	L-14689	Fledgling	Male		0.418
22-Feb-2007	19	re/or	L-14688	Juvenile	Male		3.43
24-Feb-2007	20	Y/R	L-14687	Fledgling	Male		0.194
27-Feb-2007	21	W/O	L-14653	Fledgling	Male		0.253
1-Mar-2007	22	y/bk	L-14652	Fledgling	Female		0.421
3-Mar-2007	23	r/r	L-14684	Fledgling	Male		0.193
5-Mar-2007	24	bk-bk	L14683	Fledgling	Male		0.31
5-Mar-2007	25	bk-gr	L-14682	Fledgling	Male		0.814
8-Mar-2007	26	bl/g	L-14670	Fledgling	Male		0.244
8-Mar-2007	27	bk/r	L-14655	Fledgling	Male		0.386
8-Mar-2007	28	o/bk	L-14669	Fledgling	Male		0.325
8-Mar-2007	29	g/bk	L-14668	Fledgling	Male		0.364
9-Mar-2007	30	bl-r	L-14655	Fledgling	Male		0.386
24-Mar-2007	31	Lime/ lime	L14667	Fledgling	Male		1.7
29-Mar-2007	32	or/lime	L-14666	Subadult	Male		0.368
29-Mar-2007	33	R/Lime	L-14665	Fledgling	Male		1.052
29-Mar-2007	34	Li/whi	L-14664	Fledgling	Male		0.156
30-Mar-2007	35	Y/L	L-14663	Fledgling	Male		0.165
18-Nov-2007	36	Y/R	L – 40877	Adult	Male		0.183
24-Nov-2007	37	Gr/Wh	L-40876	Nestling	Male		0.033
24-Nov-2007	38	Yellow	L-40871	Nestling	Male		0.424

Table A3.2 Kea Biochemistry

Blood Lead mg/L	UA umol/L	CK IU/L 37C	AST IU/L 37C	GGT IU/L 37C	TP g/L	ALB g/L	GLOB g/L	A/G Ratio	Ca mmol/L	Glu mmol/L	Na mmol/L	K mmol/L	Cl mmol/L
0.213	50	444	187	0	28	13	15	0.9	2.19	13.7	141	10.5	112
0.148	94	1182	195	0	26	10	16	0.63	1.98	15.3	insufficient	insufficient	insufficient
0.237	37	358	146	0	26	12	14	0.9	2.15	14.1	145	5.3	114
0.267	74	461	143	2	23	12	11	1.09	2.04	18.1	141	5.5	111
0.216	35	511	134	0	24	12	12	1	2.12	14.9	144	4.5	115
0.247	161	687	173	0	24	13	11	1.18	2.34	14.2	145	4.5	112
0.292	94	666	137	0	24	11	13	0.85	2.04	16.9	144	2.6	113
0.69	37	473	142	1	21	11	10	1.1	1.93	15.3	146	3	116
0.135	128	781	167	3	26	13	13	1	2.14	15.6	145	2.2	113
0.087	55	744	174	1	33	10	23	0.43	2.44	13	insufficient	insufficient	insufficient
0.328	157	377	125	0	28	14	14	1	2.26	14.4	141	7.6	112
0.162	167	690	153	0	26	12	14	0.86	2.12	15.6	144	3.5	110
0.23	135	491	132	2	27	14	13	1.08	2.02	23.9	150	3	113
0.028	328	580	153	1	26	13	13	1	2.07	23.7	148	2.6	110
0.293	293	513	157	3	32	15	17	0.88	2.13	19.2	153	2.5	116
0.297	35	880	147	0	27	12	15	0.8	2.2	11.8	insufficient	insufficient	insufficient
0.418	59	569	117	0	28	14	14	1	2.18	10.8	insufficient	insufficient	insufficient
3.43	49	999	154	0	26	11	15	0.73	2.11	10.8	insufficient	insufficient	insufficient

Table A3.3 Kea Haematology

Blood Lead mg/L	HCT L/L	WBC x10^9/L	Het %	Het Abs	Lymph %	Lymph Abs	Mono %	Mono Abs	Eosin %	Eosin Abs	Baso %	Baso Abs	Plasma Protein g/L
0.253	0.43	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	38
0.247	0.45	4.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	37
0.69	0.45	9.4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3.43	0.46	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.237	0.47	23.5	61	14.3	38	8.9	1	0.2	0	0	0	0	39
0.216	0.48	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	38
0.285	0.48	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	34
0.267	0.49	3.9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	37
0.23	0.49	14.4	75	10.8	18	2.6	7	1	0	0	0	0	nd
0.328	0.5	24.8	85	21.1	11	2.7	2	0.5	0	0	2	0.5	44
0.418	0.5	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.148	0.51	8.5	58	4.9	37	3.1	5	0.4	0	0	0	0	37
0.028	0.52	8.4	74	6.22	21	1.76	5	0.42	0	0	0	0	nd
0.297	0.52	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.293	0.53	16.6	84	13.9	12	2	3	0.5	0	0	1	0.2	nd
0.162	0.54	11.5	90	10.4	8	0.9	2	0.2	0	0	0	0	40
0.135	0.56	3.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	39
0.213	0.58	17	72	12.2	26	4.4	2	0.3	0	0	0	0	50

Appendix 4: Takahe Biochemistry

Serum biochemical and electrolyte analysis was obtained from takahe on Mana Island, Tiritiri Matangi Island and Kapiti Island (Table A4.1). Descriptive statistics for data from each island and for all birds combined were obtained and an analysis of variance was performed for each analyte. Post-hoc analysis was subsequently carried out for analytes that were found to have significant differences between each island. These are presented in tables A4.2 to A4.9. No correlations between biochemical analytes and blood lead levels were identified. For the purposes of this study, these were not examined further. Raw data is presented in table A4.10

Table A4.1: Biochemical and electrolyte values from takahe on Mana Island, Tiritiri Matangi Island and Kapiti Island, collected May, August and September 2007. Results from islands are presented separately and in combination despite variations as they are managed as a single population. Values with significant differences are highlighted in bold. Values are expressed as mean ± standard deviation (SD) and minimum to maximum range for all samples. Biochemical analytes reported include creatine kinase (CK), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT) and albumin/globulin ratio (A/G ratio). Values are reported in SI units.

Analyte	Tiritiri Matangi (n = 13)	Kapiti island (n = 8)	Mana Island (n = 24)	Combined (n = 45)
Uric Acid	**584.08 ±203.74**	**355.75 ±158.06**	**550.13 ±157.28**	**525.38 ± 186.58**
umol/L	**(194 – 858)**	**(172 - 591)**	**(262 – 853)**	**(172 - 858)**
CK	911.46 ±330[a]	1229.5 ± 537.41	982.21 ± 740.32	1010.12 ± 621.58[f]
IU/L 37C	(524 – 1752)	(506 – 2240)	(330 – 3636)	(330- 3636)
AST	**287.73 ± 67.04[a]**	**268.88 ± 55.48**	**348.04 ± 59.39**	**320.44 ± 67.76[f]**
IU/L 37C	**(211 – 448)**	**(181 – 362)**	**(251 – 482)**	**(181 - 482)**
GGT	**1.23 ± 0.93**	**0.38 ± 0.74**	**1.58 ± 1.18**	**1.27 ± 1.12**
IU/L 37C	**(0 – 3)**	**(0 – 2)**	**(0 – 4)**	**(0 - 4)**
Total Protein g/L	39.33 ± 2.5[b]	41.75 ± 6.27	41.46 ± 2.96	40.93 ± 3.7[g]
	(36 – 43)	(35 - 55)	(35 – 48)	(35 - 55)
Albumin	**13.82 ± 0.98[a]**	**15.75 ±1.49**	**13.08 ±1.14**	**13.77 ± 1.5[f]**
g/L	**(13 – 16)**	**(14 - 18)**	**(10 – 15)**	**(10 - 18)**
Globulins	25.73 ± 2.33[a]	26 ± 5.48	28.38 ± 2.28	27.26 ± 3.4[c] (19 -
g/L	(23 – 29)	(19 – 37)	(24 – 35)	37)
A/G ratio	**0.54 ± 0.07[a]**	**0.63 ± 0.13**	**0.46 ± 0.05**	**0.5 ± 0.09[f]**
	(0.48 – 0.67)	**(0.49-0.89)**	**(0.36 – 0.52)**	**(0.36 – 0.89)**
Calcium	**2.29 ± 0.09**	**3.21 ± 1.9**	**2.69 ± 1.52**	**2.67 ± 1.39[g]**
mmol/L	**(2.09 – 2.43)**	**(2.22 – 7.72)**	**(2.26 – 9.8)**	**(2.09 – 9.8)**
Glucose mmol/L	10.45 ± 3.18	10.79 ± 0.6	10.98 ± 2.55	10.79 ± 2.50
	(6.9 – 20)	(9.9 – 11.4)	(2.32 – 15.1)	(2.32 - 20)
Sodium mmol/L	**143.82 ± 2.89[a]**	**142.38 ± 3.02**	**147.91 ± 2.35[c]**	**145.79 ± 3.52[e]**
	(138 – 150)	**(136 – 145)**	**(144 – 152)**	**(136 - 152)**
Potassium mmol/L	**4.2 ± 0.83[a]**	**4.83 ± 0.56**	**4.05 ± 1.31[c]**	**4.24 ± 1.11[e]**
	(3 – 5.3)	**(4.2 - 5.6)**	**(2.6 – 8.9)**	**(2.6 – 8.9)**
Chloride mmol/L	108.91 ± 1.92[a]	108.5 ±2.33	108.09 ± 2.13[c]	108.38 ± 2.10[e]
	(106 – 112)	(104 – 110)	(105 – 113)	(104 – 113)
Bile Acids µmol/L	39 ± 12.93	31.13 ±8.39	-	36 ± 11.85[d]
	(21 – 61)	(17 – 43)		(17 – 61)

[a] n = 11; [b] n = 12; [c] n = 23; [d] n = 21, [e] n = 42, [f] n = 43, [g] n = 44

Table A4.2 One Way Analysis of Variance for Uric Acid (UA)

Normality Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti UA	8	0	303.000	233.000	505.500
Tiri UA	13	0	652.000	457.750	752.250
Mana UA	24	0	570.000	443.500	679.000

H = 7.534 with 2 degrees of freedom. (P = 0.023)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.023)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Tiri UA vs Kapiti UA	15.952	2.703	Yes
Tiri UA vs Mana UA	4.202	0.929	No
Mana UA vs Kapiti UA	11.750	2.191	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.3 One Way Analysis of Variance for Aspartate Aminotransferase (AST)

Normality Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti AST	8	0	262.000	237.500	304.500
Tiri AST	11	0	280.000	261.250	321.000
Mana AST	24	0	335.000	298.000	404.000

H = 11.216 with 2 degrees of freedom. (P = 0.004)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.004)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Mana AST vs Kapiti AST	14.958	2.918	Yes
Mana AST vs Tiri AST	10.947	2.394	Yes
Tiri AST vs Kapiti AST	4.011	0.688	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.4 One Way Analysis of Variance for γ-glutamyl transferase (GGT)

Normality Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti GGT	8	0	0.000	0.000	0.500
Tiri GGT	13	0	1.000	0.750	2.000
Mana GGT	24	0	2.000	1.000	2.000

H = 7.494 with 2 degrees of freedom. (P = 0.024)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.024)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Mana GGT vs Kapiti GGT	14.083	2.627	Yes
Mana GGT vs Tiri GGT	3.304	0.731	No
Tiri GGT vs Kapiti GGT	10.779	1.826	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.5 One Way Analysis of Variance for Albumin

Normality Test: Passed (P = 0.059)

Equal Variance Test: Passed (P = 0.283)

Group	Name	N	Missing	Mean	Std Dev	SEM
Kapiti	ALB	8	0	15.750	1.488	0.526
Tiri	ALB	11	0	13.818	0.982	0.296
Mana	ALB	24	0	13.083	1.139	0.232

Source of Variation	DF	SS	MS	F	P
Between Groups	2	42.705	21.352	15.538	<0.001
Residual	40	54.970	1.374		
Total	42	97.674			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 0.999

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Sig?
Kapiti ALB vs. Mana ALB	2.667	5.572	0.00000189	0.017	Yes
Kapiti ALB vs. Tiri ALB	1.932	3.546	0.00101	0.025	Yes
Tiri ALB vs. Mana ALB	0.735	1.722	0.0929	0.050	No

Table A4.6 One Way Analysis of Variance for Albumin: Globulin Ratio

Normality Test: Passed (P = 0.093)

Equal Variance Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti A/G	8	0	0.625	0.530	0.660
Tiri A/G	11	0	0.540	0.480	0.600
Mana A/G	24	0	0.470	0.445	0.500

H = 21.057 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Kapiti A/G vs Mana A/G	21.146	4.125	Yes
Kapiti A/G vs Tiri A/G	7.369	1.263	No
Tiri A/G vs Mana A/G	13.777	3.013	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.7 One Way Analysis of Variance for Calcium (Ca)

Normality Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti CAL	8	0	2.380	2.295	3.195
Tiri CAL	12	0	2.315	2.255	2.345
Mana CAL	24	0	2.390	2.320	2.440

H = 7.828 with 2 degrees of freedom. (P = 0.020)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.020)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Mana CAL vs Tiri CAL	12.250	2.697	Yes
Mana CAL vs Kapiti CAL	0.417	0.0795	No
Kapiti CAL vs Tiri CAL	11.833	2.018	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.8 One Way Analysis of Variance for Sodium (Na)

Normality Test: Passed (P = 0.317)
Equal Variance Test: Passed (P = 0.694)

Group Name	N	Missing	Mean	Std Dev	SEM
Kapiti NA	8	0	142.375	3.021	1.068
Tiri NA	11	0	143.818	2.892	0.872
Mana NA	24	1	147.913	2.353	0.491

Source of Variation	DF	SS	MS	F	P
Between Groups	2	239.734	119.867	17.357	<0.001
Residual	39	269.337	6.906		
Total	41	509.071			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Bonferroni t-test):
Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Mana NA vs. Kapiti NA	5.538	5.134	<0.001	Yes
Mana NA vs. Tiri NA	4.095	4.251	<0.001	Yes
Tiri NA vs. Kapiti NA	1.443	1.182	0.733	No

Table A4.9 One Way Analysis of Variance for Potassium (K)

Normality Test: Failed (P < 0.050)
Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
Kapiti K	8	0	4.700	4.350	5.350
Tiri K	11	0	4.600	3.600	4.875
Mana K	24	1	3.700	3.500	4.175

H = 8.951 with 2 degrees of freedom. (P = 0.011)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.011)

To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
Kapiti K vs Mana K	14.829	2.945	Yes
Kapiti K vs Tiri K	8.847	1.552	No
Tiri K vs Mana K	5.982	1.330	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Table A4.10 Takahe Raw Data - Blood lead, PCV and Biochemistry from Mana, Tiritiri and Kapiti Islands

Island	Sample mumber	Bld Pb mg/L	PCV	UA umol/L	CK IU/L	AST IU/L	GGT IU/L	TP g/L	Alb g/L	Glob g/L	A/G ratio	Cal mmol/L	Glu mol/L	Na mmol/L	K mmol/L	Cl mmol/L	BA umol/L
Mana	1	0.012	0.39	262	1008	292	2	40	13	27	0.48	2.37	10.4	144	4.2	107	n/d
	2	0.007	0.39	271	731	286	1	41	13	28	0.46	2.3	10	148	3.5	108	n/d
Chick	3	0.116	0.42	339	2409	328	1	35	11	24	0.46	2.39	14	146	3.5	105	n/d
	4	0.06	0.40	451	456	323	1	42	13	29	0.45	9.8	2.32	148	4.1	108	n/d
Chick	5	0.007	0.44	488	1589	410	0	41	12	29	0.41	2.37	10.8	146	4.8	110	n/d
	6	0.007	0.44	374	738	407	0	41	14	27	0.52	2.26	13.7	151	3.4	110	n/d
	7	0.011	0.40	551	736	292	2	44	14	30	0.47	2.47	11.1	149	2.7	105	n/d
	8	0.006	0.40	484	647	284	2	41	14	27	0.52	2.36	11.8	150	2.6	107	n/d
	9	0.008	0.44	660	1029	339	1	44	15	29	0.52	2.49	11.1	147	4.1	105	n/d
	10	0.008	0.40	697	713	281	2	43	14	29	0.48	2.43	12.6	144	3.5	105	n/d
	11	0.012	0.36	735	905	373	0	38	10	28	0.36	2.3	9.1	n/d	n/d	n/d	n/d
	12	0.005	0.42	853	442	340	0	41	14	27	0.52	2.29	9.2	145	6.4	107	n/d
Chick	13	0.005	0.36	701	330	439	0	48	13	35	0.37	2.26	8.2	146	8.9	108	n/d
	14	0.007	0.39	623	640	331	2	38	13	25	0.52	2.31	11.2	149	4.2	112	n/d
	15	0.001	0.44	476	575	404	2	42	13	29	0.45	2.39	11.3	152	4	113	n/d
Chick	16	0.007	0.36	642	796	355	2	37	12	25	0.48	2.45	9.7	149	4.7	109	n/d
	17	0.003	0.38	589	1142	304	2	39	12	27	0.44	2.33	11.2	148	3.4	109	n/d
	18	0.008	0.40	662	569	404	1	40	13	27	0.48	2.34	11	147	4	107	n/d
	19	0.007	0.39	679	1875	430	3	41	13	28	0.46	2.39	9	147	3.5	107	n/d
	20	0.012	0.37	679	635	482	4	44	12	32	0.38	2.45	11.4	146	4	109	n/d
	21	0.017	0.37	683	3636	366	4	47	14	33	0.42	2.4	13.6	147	3.6	107	n/d
	22	0.007	0.34	436	820	317	3	41	14	27	0.52	2.39	11.8	151	3.7	109	n/d
	23	0.015	0.32	497	569	251	1	44	14	30	0.47	2.5	13.8	152	3.5	109	n/d
	24	0.008	0.38	371	583	315	2	43	14	29	0.48	2.43	15.1	150	2.9	110	n/d

Table A4.10 Continued

Island	Sample mumber	Bld Pb mg/L	PCV	UA umol/L	CK IU/L	AST IU/L	GGT IU/L	TP g/L	Alb g/L	Glob g/L	A/G ratio	Cal mmol/L	Glu mol/L	Na mmol/L	K mmol/L	Cl mmol/L	BA umol/L
Tiritiri	25	0.012	0.40	342	1065	244	0	37	14	23	0.61	2.24	9.4	142	4.8	108	39
	26	0.012	0.47	756	1018	331	2	37	14	23	0.61	2.35	7.7	143	4.7	107	45
	27	0.015	0.42	703	549	211	1	43	15	28	0.54	2.36	10.5	150	3	111	39
	28	0.148	0.45	858	524	261	2	37	13	24	0.54	2.18	10.9	144	3.7	109	35
	29	0.111	0.42	652	807	262	1	40	13	27	0.48	2.31	12.5	145	3	108	22
Chick	30	0.107	0.42	751	713	280	3	40	13	27	0.48	2.28	10.3	144	3.6	109	50
	31	0.058	0.42	495	935	275	0	40	13	27	0.48	2.32	6.9	144	5.3	107	21
	32	0.023	0.47	793	941	383	1	42	14	28	0.5	2.34	20	143	3.6	110	48
	33	0.02	0.42	702	929	289	2	36	13	23	0.57	2.09	9.6	143	5	112	61
	34	0.007	0.47	503	793	291	1	40	16	24	0.67	2.42	9.2	146	4.6	111	58
	35	0.015	0.46	194	1752	448	1	43	14	29	0.48	2.32	9.7	138	4.9	106	32
	36	0.028	0.38	498	n/d	n/d	0	37	n/d	n/d	n/d	2.27	9.7	n/d	n/d	n/d	25
	37	0.011	0.42	346	n/d	n/d	2	n/d	n/d	n/d	n/d	n/d	9.4	n/d	n/d	n/d	32
Kapiti	38	0.03	0.41	172	1045	263	0	36	17	19	0.89	2.34	11	145	5.5	106	29
	39	0.011	0.38	499	1523	314	0	42	16	26	0.62	3.93	10.8	143	5.6	110	35
	40	0.006	0.40	284	735	295	2	41	14	27	0.52	2.37	11.4	144	4.4	108	23
	41	0.028	0.42	591	997	362	0	43	15	28	0.54	2.46	9.9	143	4.3	110	43
	42	0.032	0.39	186	1353	181	1	55	18	37	0.49	7.72	11.4	136	4.5	104	29
	43	0.024	0.43	322	1437	261	0	38	15	23	0.65	2.25	11.2	143	4.9	110	38
	44	0.016	0.49	280	2240	251	0	44	17	27	0.63	2.39	10.7	140	5.2	110	35
	45	0.007	0.44	512	506	224	0	35	14	21	0.67	2.22	9.9	145	4.2	110	17

Appendix 5: Takahe Tissue Lead Levels

Table A5.1 Lead Levels in Formalin-fixed Kidney or Liver from Island Takahe

ACCN #	Location	Sex	Age	Tissue	Pb Value
23816	Mana/kapiti	Female	8 years	Kidney	0.075
28395	Mana	Male	10 Years	Kidney	0.11
28670	Mana	Male	7 Years	Kidney	0.054
29028	Maud	Female	7 Years	Kidney	0.032
29057	Kapiti	Male	12.5 Years	Kidney	0.67
29088	Mana	Male	Juvenile	Kidney	0.28
29944	Maud	Female	15 Years	Kidney	0.044
30382	Tiritiri	Female	2 Years 6 Months	Kidney	0.064
30545	Mana	Male	Mature	Kidney	0.013
30805	Maud	Male	17 years	Kidney	0.12
31956	Maud	Unknown	38 Days	Kidney	0.0078
31964	Kapiti	Male	8 Weeks	Kidney	0.044
33395	Maud	Male	Adult	Kidney	0.024
34299	Maud	Male	4 Years	Kidney	0.037
36089	Kapiti	Unknown	Unknown	Kidney	0.12
36090	Kapiti	Male	16 Years	Kidney	0.44
37452	Mana	Male	Juvenile	Kidney	0.047
38435	Maud	Male	1 Year	Kidney	0.037
38442	Mana	Female	13 Years	Kidney	0.042
38485	Mana	Male	Juvenile	Kidney	0.05
38028	Maud	Unknown	Adult	Liver	0.18

Table A5.2 Lead Levels in Frozen Kidney or Liver from Murchison Mountain Takahe

PM number	Tissue	Pb Value
39590	Kidney	0.017
39605	Kidney	0.019
39606	Kidney	0.36
39611	Kidney	0.042
39619	Kidney	0.010
39622	Kidney	0.017
39635	Kidney	0.016
39711	Kidney	0.031
39728	Kidney	0.026
39734	Kidney	0.033
39747	Kidney	0.15
39748	Kidney	0.14
39750	Kidney	0.095
39751	Kidney	0.034
39752	Kidney	0.023
39756	Kidney	0.025
39767	Kidney	0.074
39769	Kidney	0.037
39771	Kidney	0.023
39772	Kidney	0.013
39613	Liver	0.056
39620	Liver	0.078
39623	Liver	3.9
39652	Liver	0.028
39770	Liver	0.035
39771	Liver	0.047
39772	Liver	0.067

Appendix 6. Diagnostic and Treatment Protocol for Harriers Admitted to the New Zealand Wildlife Health Centre

Patient Number:
Clinician:

Day 1(date):

History:

Clinical exam:
Weight:

Neurological exam (see attached)

Diagnostics:
- Radiographs:
- Bloods:
  - CBC £
  - Biochemistry £
  - Uric acid £
  - Blood lead £
  - Cholinesterase £
- EMG (contact Craig Johnson ext:7285) £

IV catheter placed Y/N Leg: Left/Right

If clinical signs suggest lead poisoning:
- CaEDTA 50mg/kg Dose:______________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:_________________ IV/PO £ am £ pm
- Food offered _____________________________ £ am £ pm

Other treatment:

Day 2 (date):

Weight:
Eaten: Y/N
Cast:
Treatment
- CaEDTA 50mg/kg Dose:______________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:_________________ IV/PO £ am £ pm
- Food offered _____________________________ £ am £ pm

Other treatment:

Day 3 (date):

Weight:
Eaten: Y/N
Cast:
Treatment

- CaEDTA 50mg/kg Dose:______________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:__________________ IV/PO £ am £ pm
- Food offered ______________________________ £ am £ pm

Other treatment:

Day 4 (date):

Weight:
Eaten: Y/N
Cast:
Treatment

- CaEDTA 50mg/kg Dose:______________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:__________________ IV/PO £ am £ pm
- Food offered ______________________________ £ am £ pm

Other treatment:

Day 5 (date):

Weight:
Eaten: Y/N
Cast:
Treatment

- CaEDTA 50mg/kg Dose:______________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:__________________ IV/PO £ am £ pm
- Food offered ______________________________ £ am £ pm

Other treatment

Day 6 (date):

Weight:
Eaten: Y/N
Cast:
Treatment:

- Stop CaEDTA
- LRS 75ml/kg Dose:___________________IV/PO £ am £ pm
- Food offered ______________________________ £ am
- Other treatment: ***NPO overnight***

Day 7 (date):

Weight:
Eaten: Y/N
Cast:
Diagnostics:

- Radiographs if indicated:
- Bloods:
    - CBC £
    - Uric acid £
    - Blood lead £
    - Cholinesterase £

IV catheter changed Y/N Leg: Left/Right
Medication Doses reassessed: £

Treatment

- LRS 75ml/kg Dose:_________________ IV/PO £ am £ pm

Other treatment:

Day 8 (date):

Weight:
Eaten: Y/N
Cast:
Treatment

- CaEDTA 50mg/kg Dose:_____________ IV/IM £ am £ pm
- LRS 75ml/kg Dose:_________________ IV/PO £ am £ pm

Other treatment:

Appendix 7. Avian Neurological Exam

(modified for wild harriers with a suspect lead neuropathy)

Clinic Number:
Clinician

DISTANCE EXAMINATION

1. **Posture and Gait**

- Any weakness or ataxia Y N
- Normal symmetrical, upright stance Y N
- Base wide stance Y N
- Head tilt Y N

2. **Mental status**

- Degree of consciousness Normal Depressed Hyperactive
- Normal responses Y N
- Normal activity Y N
- Convulsions or Syncope Y N

3. **Cranial Nerve Examination (circle if sign of dysfunction. N/A if not assessed)**

I Loss of appetite, cannot locate food Normal
Abnormal

II hesitant walking, poor obstacle avoidance, anisocoria Normal
Abnormal

V *Motor*: loss of ability to chew or use beak to climb Normal
Abnormal

VIII Loss of hearing, poor response to sound. Normal
Abnormal

IX Dysphagia Normal
Abnormal

X Dysphagia, including oesophagus and crop related Normal
Abnormal

4. **Vestibular system:**

(Includes CN III, IV, VI, VIII, brain stem and cerebellum)

- Head tremor or incoordination Y N
- Head tilt on ipsilateral side Y N
- Spontaneous nystagmus Y N
- Balance loss Y N
- Strabismus Y N

Comments:

IN-HAND EXAMINATION

1. **Posture and Gait**	**Right**		**Left**	
• Proprioceptive deficits	Y	N	Y	N

2. **Cranial Nerve Examination**

II Poor menace response, Normal

Abnormal

III Abnormal eye movements, anisocoria, ventrolateral strabismus Normal

Abnormal

IV Lateral rotation of eye (difficult to assess) Normal

Abnormal

V *Sensory*: loss of response to touching each side of the face Normal

Abnormal

VI Medial strabismus, change in 3rd eyelid position Normal

Abnormal

VII Poor menace response, poor blink response lateral canthus Normal

Abnormal

XII Atrophy or deviation of tongue Normal

Abnormal

Comments:

3. **Pupillary Light Reflexes.**	**Right**		**Left**	
• Normal direct pupil response to light	Y	N	Y	N
• Normal Consensual response to light	Y	N	Y	N

Comments:

4. Wing Function

• Normal muscle strength and tone	Y	N	Y	N
• Loss of strong wing flexion (wingbeat)	Y	N	Y	N
• Muscle atrophy	Y	N	Y	N
• Asymmetrical wing carriage	Y	N	Y	N
• Wing withdrawal	Y	N	Y	N
• Detectable fracture	Y	N	Y	N

Comments:

5. **Leg Function**	**Right**		**Left**	
• Normal muscle strength and tone	Y	N	Y	N
• Muscle atrophy – proximal muscles	Y	N	Y	N
• Muscles atrophy- distal muscles	Y	N	Y	N
• Cutaneous touch/pain dorsal foot	Y	N	Y	N
• Cutaneous touch/pain palmar foot	Y	N	Y	N
• Cutaneous touch/pain dorsal leg	Y	N	Y	N
• Cutaneous touch/pain caudal leg	Y	N	Y	N
• Cutaneous touch/pain medial leg	Y	N	Y	N
• Central registration of pain	Y	N	Y	N
• Withdrawal reflex	Y	N	Y	N
• Crossed extensor reflex	Y	N	Y	N
• Grasping reflex	Y	N	Y	N

- Knuckling of digit Y N Y N
- Patella reflex (stretch) Y N Y N
- Detectable fracture Y N Y N

Comments:

6. Cloacal Function

- Loss of response to pinch or pinprick Y N
- Soiling of vent and tail feathers Y N

Comments:

7. Other

- Tail Flick Normal

  Abnormal

Comments:

Appendix 8. Raw Harrier Data

Table A8.1 Harrier Blood Lead Concentrations.

Number	Blood lead mg/L	Lab	Comments	Other
29971	1	Lab	Lead not clenched	
34500	3.1	Lab	Clenched	
35112	0.1	Lab	Control	
35118	2.3	Lab	Clenched	
35136	0.3	Lab	Clenched	bld lead 5 hours post CaEDTA
35357	1.5	Lab	Clenched	
35409	0.1	Lab	Control	
35445	0.2	Lab		Lab Error Blood Pb
35452	3.7	Lab	Clenched	
35537	0.1	Lab		
35597	0.1	Lab		
35648	0.5	Lab	Clenched	
35743	0.1	Lab		
35794	3	Lab	Clenched	
35899	0.1	Lab	Control	
36053	0.4	Lab	Lead not clenched	Exclude*
36090	0.2	Lab	Clenched	Lab Error Blood Pb
36736	0.7	Lab	Lead not clenched	
36890	0.4	Lab	Lead not clenched	Exclude*
36917	0.1	Lab	Control	
37857	0.1	Lab		
38062	Hi	LeadCare	Lead not clenched	Bld sent to lab but lab error
38096	0.014	LeadCare		
38137	Hi	LeadCare	Clenched	No actual Pb value obtained
38311	0.098	LeadCare		
38354	2.5	Lab	Lead not clenched	2.82 mg/L leadcare
38402	0.051	LeadCare	neuro	
38576	0.014	LeadCare		
38712	0.04	LeadCare		
none	0.021	LeadCare		

* Excluded from lead not clenched analysis as diagnosis + protocol not followed

Table A8.2 Clenched Feet Harrier Haematology

Blood Pb	Serum cholinesterase	HCT L/L	WBC x10^9/L	Band Het %	Band Het abs	Het %	Het abs	Lymph%	Lymph abs	Mono%	Mono abs	Eos %	Eos abs	Baso %	Baso abs	TP g/L
0.2	1.8	0.36	3.5	2	0.1	79	2.8	9	0.3	10	0.4	0	0	0	0	52
0.5	n/d	0.37	18.8	0	0	90	16.9	2	0.4	8	1.5	0	0	0	0	46
1.5	n/d	0.3	12.2	1	0.1	84	10.2	13	1.6	2	0.2	0	0	0	0	42
3	0.4	0.33	11.7	16	1.9	61	7.1	7	0.8	9	1.1	9	1.1	0	0	39
3.1	n/d	0.31	27.2	5	1.4	91	24.8	2	0.5	2	0.5	0	0	0	0	44
3.7	0.3	0.38	14.8	2	0.3	80	11.8	14	2.1	4	0.6	0	0	0	0	53

Table A8.3 Lead, No Clenched Feet Harrier Haematology

Blood Pb	Serum cholinesterase	HCT L/L	WBC x10^9/L	Band Het %	Band Het abs	Het %	Het abs	Lymph%	Lymph abs	Mono%	Mono abs	Eos %	Eos abs	Baso %	Baso abs	TP g/L
0.7	n/d	0.37	3.2	3	0.1	86	2.8	2	0.1	9	0.3	0	0	0	0	32
2.82	0.3	n/d	12.25	0	0	72		17		5		2		4		n/d
Hi	0.3	0.39	11.5	0	0	56	6.4	28	3.2	7	0.8	4	0.5	5	0.6	33
1	n/d	0.49	7.9*	0	0	79	6.2	18	1.4	3	0.2	0	0	0	0	60

Table A8.4 Control Harrier Haematology

Blood Pb	Serum cholinesterase	HCT L/L	WBC x10^9/L	Band Het %	Band Het abs	Het %	Het abs	Lymph%	Lymph abs	Mono%	Mono abs	Eos %	Eos abs	Baso %	Baso abs	TP g/L
<0.1	n/d	0.39	19	0	0	82	16	9	1.7	4	0.8	3	0.6	2	0.4	42
<0.1	n/d	0.37	21.3	1	0.2	93	20	4	0.9	2	0.4	0	0	0	0	39
<0.1	n/d	0.34	15.3	0	0	93	14	5	0.8	2	0.3	0	0	0	0	32
<0.1	n/d	0.46	27.3	0	0	78	21	3	0.8	5	1.4	14	3.8	0	0	47

Table A8.5: Clenched Feet Harrier Biochemistry

UA	CK	AST	GGT	TP	ALB	GLOB	A/G	Cal	Glu	Na	K	Cl
856	896	453	3	32	14	18	0.8	2.62	19.2	154	2	116
243	1097	218	2	42	19	23	0.83	2.89	18.3	155	1.9	118
156	1299	347	3	46	18	28	0.64	2.88	17.3	160	2	124
525	1297	454	2	37	13	24	0.54	2.66	17.5	162	3.2	134
1023	752	738	11	30	14	16	0.88	2.34	20.4	157	2.4	120
291	477	308	3	45	15	30	0.5	2.68	20.6	152	2.5	122
168	905	311	3	41	14	27	0.52	2.78	18.9	155	2.9	121

Table A8.6 Lead, Non-clenched Feet Harrier Biochemistry

UA	CK	AST	GGT	TP	ALB	GLOB	A/G	Cal	Glu	Na	K	Cl
1041												
802	1343	207	1	27	8	19	0.42	2.51	17.6	157	3.7	130
153	767	471	0	25	11	14	0.8	2.4	18.2	152	3.5	121

Table A8.7 Control Harrier Biochemistry

UA	CK	AST	GGT	TP	ALB	GLOB	A/G	Cal	Glu	Na	K	Cl
166	966	1256	4	33	13	20	0.65	2.52	21	161	3	125
335	708	535	1	32	13	19	0.68	2.48	19	154	3.6	125
503	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
233	708	245	2	34	16	18	0.89	2.39	18.2	155	1.9	122