VALIDATION OF A PROTHROMBIN TIME (PT) ASSAY FOR ASSESSMENT OF BRODIFICOUM EXPOSURE IN JAPANESE QUAIL AND BARN OWLS

by

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ABSTRACT

Brodifacoum is an anticoagulant rodenticide which has been linked to secondary poisoning of wild birds. The prothrombin time (PT) and activated clotting time (ACT) tests measure the response of the extrinsic and intrinsic coagulation pathways, respectively. The ACT, hemoglobin, hematocrit and PT measured in Japanese quail (*Coturnix japonica)* were exposed to brodifacoum at 0, 0.8, 1.4, 1.9, and 2.5 mg/kg and sampled 1, 3, 5 and 7 days post-exposure. Free-living barn owl (*Tyto alba*) PTs were also measured. The ACT increased with exposure, but showed no clear dose-response relationship. Hemoglobin and hematocrit decreased dose- and time-dependently at doses ≥1.4 mg/kg with no significant change at 0.8 mg/kg. PTs were significantly prolonged in a dose- and time-dependent manner at all doses. Although the ACT test requires further evaluation, PTs appear to be a useful indicator of avian anticoagulant rodenticide exposure suggesting that the barn owls sampled were not significantly exposed.

Keywords: Brodifacoum, prothrombin time, rodenticide biomonitoring, barn owl

DEDICATION

To my husband,

my family, my dear friends, and the birds.

ACKNOWLEDGEMENTS

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CHAPTER 1: THESIS INTRODUCTION

1.1 Rodent control in agriculture and conservation

Control of mammalian, particularly rodent, pests has posed a challenge to humans since we began to store food and other palatable or useful items. More recently, introduced and sometimes invasive mammals have created conservation challenges, particularly on islands, along with ethical concerns of eradication for the sake of protecting biodiversity and human resources (Atkinson, 2001; Warburton and O'Connor, 2004; Littin and Mellor, 2005; Meerburg et al., 2008). Methods used to eradicate those invasive species, range from individuals being shot or trapped, to introduction of biological control species, to entire areas being covered with poison baits. These efforts may result in harmful effects on the ecosystem, such as when a biological control organism becomes invasive itself, or when non-target native animals are incidentally poisoned. If continued attempts are to be made to control those invasives, perhaps more diligent monitoring of the impacts on native species is required.

Rodents constitute 42% of the known mammalian species (MacDonald, 2001) including some of the most important pest species worldwide (Singleton et al., 1999). Species of most concern include *Rattus norvegicus* (Norway or brown rat), *R. rattus* (Roof or black rat), and *Mus musculus* (house mouse), as well as

various species of burrowing rodents such as ground squirrels, gophers and voles. As rodents are attracted to agricultural and populated areas in search of food and nesting habitat, their high reproductive rates cause population explosions and their gnawing capabilities are responsible for property damage, the consumption of growing crops and spoiling of stored food products. Contamination of food products in restaurants, food manufacturing, packing and storage facilities with urine, feces and hair may lead to the transmission of bacterial, protozoal, or viral diseases, ranging in severity from the bacterial disease of leptospirosis to serious viral infections caused by Hantavirus (Schmaljohn and Hjelle, 1997; CDC, 2006). Rodents can also be responsible for indirect losses in agriculture, e.g. California ground squirrels not only compete with cattle for forage but also burrow holes in the landscape, potentially leading to injury of cattle (Howard et al., 1959) as well as transferring disease to livestock (Kijlstra et al., 2008). In the United States, losses due to the destruction of stored grain and other damages by rats are estimated at USD$19 billion per year excluding costs of rodent control measures (Pimentel et al., 2005) and in Britain, the cost of rodent damage to farms is estimated at £10-20 M per year (Elliott, 1995).

In addition to agricultural problems, rodents are a challenge for conservation efforts worldwide (Howald et al., 2007) and pose a serious threat to biodiversity on many islands. Non-native rats and mice have invaded more than 80% of island groups (Atkinson, 1985). Island ecosystems are believed to be vulnerable to invasive mammals because flora and fauna have evolved in the

absence of terrestrial mammals, and many island species lack defence mechanisms against mammalian predation and herbivory (Jones et al., 2006). In fact, 83% of recent avian and mammalian extinctions worldwide occurred on islands (Aguirre-Munoz et al., 2008) with at least 92% of bird extinctions over the last 400 years being island nesters (Kress, 1998). Currently, 44% of currently threatened taxa are on islands (Aguirre-Munoz et al., 2008) and two-thirds of all currently threatened birds nest on islands (Kress, 1998). Jones et al. (2008) identified 115 independent rat-seabird interactions on 61 islands or island chains involving 75 seabirds in 10 families. Of the three rodent species introduced, *Rattus rattus* had the largest impact followed by *R. norvegicus* and *R. exulans* with seabirds of the family Hydrobatidae and other small burrow-nesting seabirds being most affected and birds of the family Laridae and other larger birds being the least vulnerable. Birds that are burrow or crevice nesters may be particularly vulnerable to introduced rats due to similar preferences for habitat which may result in direct predation of eggs, chicks and adults, as well as indirect effects caused by disturbance of nest sites and mate loss during the breeding season (Jones et al., 2006).

Until the early twentieth century, rodent control included primarily arsenic, traps, and use of natural predators such as dogs, ferrets, and falcons (Elliott, 1995). With increasingly concentrated human populations worldwide, a corresponding increase in food and waste production, and technological advances in chemistry and pharmacology, rodent control has evolved to include an arsenal of chemicals. The benefit of acutely lethal rodenticides is their rapid

action, however this frequently works as a disadvantage when sub-lethal quantities are consumed and symptoms deter further bait consumption, leading to suffering and possibly recovery of the target animal. It is now considered that the most effective rodenticides have three main qualities: 1) they delay symptom onset to avoid creating bait shyness; 2) they are highly toxic with low LD_{50}s to avoid sub-lethal quantities being consumed; and 3) they are easily incorporated into environmentally stable formulations (Elliott, 1995).

A range of anticoagulant rodenticides were introduced in the 1940s which largely met these criteria. Anticoagulant rodenticides are relatively slow-acting compounds and symptoms are not typically experienced for at least 3 days, which is normally after a lethal dose has been consumed, thereby preventing the development of bait aversion (Pelfrene, 2001). These rodenticides are also fairly potent, with LD_{50} values from 0.2 mg/kg to 100 mg/kg depending on the compound and species. Fortunately, vitamin K can be used at high doses as an antidote in the case of accidental anticoagulant poisoning, allowing some protection of non-target animals and humans (Whitlon et al., 1978). The two families of anticoagulants used as rodenticides are the coumarins and the indanediones. Coumarins include warfarin, coumatetralyl, difenacoum, difethialone, bromadiolone, and brodifacoum, while indanediones include diphacinone and chlorophacinone. Not long after anticoagulant rodenticides were introduced, resistance to warfarin and some warfarin derivatives was discovered in several rodent populations in Europe (Boyle, 1960; Lund, 1964; Greaves and Ayres, 1967), Canada (Cronin, 1979) and the US (Jackson and Kaukeinen, 1972;

Franz and Padula, 1980), prompting investigation into more effective compounds (Lund, 1984). In 1975, several coumarin derivatives were discovered to be very effective against warfarin-resistant rats (Hadler and Shadbolt, 1975). A number of these compounds, including brodifacoum, were soon developed into commercial formulations now known as 'second-generation anticoagulant rodenticides' (SGARs).

A variety of formulations exist for each SGAR in order to customize the rodenticide baits for specific environments and species. These bait types range from solid pellets and paraffin blocks to tracking powder and liquid concentrates (PMRA, 2004). In British Columbia (BC), Canada, over 34,000 kilograms of rodenticide bait was used in 2003 with the anticoagulant rodenticides comprising 81% of the total quantity (see Table 1). Brodifacoum was the third most widely used active ingredient in BC and in the United States (see Table 1). Due to the high potency of brodifacoum compared to other rodenticides, when the number of over-the-counter placement units is compared, it was the brodifacoum-based baits which were most commonly sold (Kaukeinen et al., 2000). From 2002 to 2003, brodifacoum exports from the US increased by 60% while difethialone exports decreased by 76% (Smith et al., 2008).

Numerous rodent eradication programs using anticoagulant rodenticides, mostly brodifacoum, have been conducted in agricultural areas and in small communities (Kaukeinen and Rampaud, 1986; Saunders et al., 2007). Rodents have been eradicated from at least 284 islands worldwide with 90% of the 387 reported attempts being successful (Howald et al., 2007). Brodifacoum has been

used in 91% of total area of islands cleared of invasive rodents and in 71% of all successful eradications (Howald et al., 2007). Most eradications occurred in Australasia with a large proportion in New Zealand (Towns and Broome, 2003). On islands off western Mexico, five black rat, one house mouse, and six European rabbit eradications were successful at an estimated cost of $58 USD per hectare per invasive species (Aguirre-Munoz et al., 2008). Eradication attempts have occurred primarily on islands of <100 hectares which lack non-target vertebrates (Howald et al., 2007). However, successful eradications have also been carried out on the large subantarctic Campbell Island (11,300 ha) (Towns and Broome, 2003) and on the Californian Anacapa island containing endemic deer mice (Jones et al., 2006). One study monitoring the nesting habits of Xantu's murrelets before and after rat eradication showed an increase in nest survival from 4% to 77%, affirming that removing rats may provide a benefit to seabird populations (Jones et al., 2006).

1.2 Brodifacoum toxicology

1.2.1 Chemical properties

Brodifacoum (CAS no. 56073-10-0; Figure 1.1) is a derivative of ^{2}H-1-benzopyran-2-one (coumarin) with a long non-polar side-chain attached at the carbon-3 position of the coumarin moiety. Brodifacoum is hydrolytically stable (at pH 5, 7 and 9), has very low volatility ($<1.33 \times 10^{-7}$ Pa), is insoluble in water (<10 mg/L), is relatively tasteless and odourless (WHO, 1995), and has a log octanol-water partition coefficient (Log K_{ow}) of 8.5 (PMRA, 2004). These chemical properties allow brodifacoum to be incorporated into bait formulations palatable

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to the target rodent and to be stable under a variety of environmental conditions. Brodifacoum is one of the most widely used SGARs and is registered in over 40 countries (Kaukeinen and Rampaud, 1986).

1.2.2 Toxicity

Numerous studies have been conducted to determine the efficacy of brodifacoum at killing rodents, including warfarin-resistant rats. Brodifacoum is highly toxic, with an LD_{50} in rats and mice after a single oral dose of 0.27 and 0.4 mg/kg, respectively, with cats and dogs having an LD_{50} of 0.25 mg/kg (WHO, 1995). Avian species vary significantly in their susceptibility to brodifacoum with oral LD_{50}s ranging from <1 mg/kg to > 20 mg/kg (WHO, 1995). The pathology of brodifacoum in birds has not been well documented and limited data are available as to the lowest concentration which will cause a sub-lethal effect (Howald, 1997; Bailey et al., 2005).

1.2.3 Absorption, distribution, metabolism and elimination

There is extensive information on the pharmacokinetics of warfarin resulting from medical usage, and studies of warfarin resistance in rodents. As a result, where brodifacoum specific data is lacking, I have used knowledge available for warfarin. Brodifacoum is absorbed across the gastrointestinal tract, through inhalation, and to a lesser extent across the skin (WHO, 1995). Metabolism in the digestive tract likely varies based on the abundance and type of bacterial species present (Poche and Mach, 2001) but does not appear to be significant. Once warfarin enters the bloodstream, it becomes bound to plasma

proteins (Ikeda et al., 1968). Warfarin and metabolites accumulate primarily in the liver, but are also detected in the kidneys and pancreas (Link et al., 1965). Laboratory studies examining the pharmacokinetics of brodifacoum in rats have focused on its biphasic elimination from the liver, with a short rapid phase followed by a long slow elimination phase (Parmar and Batten, 1987; Parmar et al., 1987). Hepatic half-lives in the rat are reported to range from 25 to 350 days depending on the amount and number of doses given, however > 130 days is generally accepted (Bachmann and Sullivan, 1983; Godfrey, 1985; Parmar et al., 1987; WHO, 1995; Fisher et al., 2003; US EPA, 2004). The plasma half-life of brodifacoum in rats was 156 hours with a liver:plasma ratio of 21.2:1 (Bachmann and Sullivan, 1983). In rabbits, brodifacoum dosing resulted in a bi-exponential decay of plasma concentrations and a terminal half-life of 60 hours (Breckenridge and L'E Orme, 1972).

Limited research has been conducted investigating the metabolism of brodifacoum in terms of the enzymatic pathways involved and the metabolites produced. However, numerous studies have examined the hepatic metabolism of warfarin by measuring metabolites eliminated from the rat after exposure and using *in vitro* studies with human microsomes, and brodifacoum is assumed to undergo similar metabolism. Elimination of warfarin occurs primarily through the feces and urine (WHO, 1995) with metabolites in the urine including 4-, 6-, 7-hydroxywarfarins, glucuronidated 7-hydroxywarfarin and some parent compound. Metabolism by hepatic rat microsomes produced 6-, 7-, and 8-hydroxycoumarins (Ikeda et al., 1968) and human microsomal metabolism resulted in primarily 6-,7-,

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8-, and 10-hydroxywarfarins (Kaminsky and Zhang, 1997). Phase II metabolites of warfarin that are produced in rat hepatocytes include a mixture of glucuronides and sulfate conjugates (Jansing et al., 1992).

1.2.4 Coumarin mode of action

Much of the research into how coumarins disrupt coagulation has focused specifically on warfarin in mammalian models in order to contribute to therapy of coagulation diseases in humans or to understand warfarin resistance in rats. However, that knowledge may be applied more broadly to understanding the mechanisms of action for all coumarins. Coagulation in mammals occurs as a response to external tissue damage and internal disruptions of the blood vessel. When tissue damage occurs, a tissue factor (TF) is released by endothelial cells in the blood vessel which activates coagulation factor VII (FVII) forming the extrinsic pathway. When injury occurs within the blood vessel and collagen is exposed, thrombocytes (platelets) aggregate at the site to obstruct blood flow, while coagulation factor XII (FXII) is activated and initiates the intrinsic pathway (Michal, 1999; Figure 1.2). Both of these cascade-activating pathways then lead to a common pathway through the activation of factor X, the conversion of prothrombin to thrombin, and the transformation of soluble fibrinogen to an insoluble fibrin clot (Michal, 1999; Figure 1.2).

Many of the proteins involved in coagulation are manufactured in the liver and are subsequently released into the bloodstream where they interact in a proteolytic pathway for signal amplification and the production of a rapid response to injury in the form of a blood clot. During the production of several of

these proteins, vitamin K hydroquinone is required as a cofactor for gamma-glutamyl carboxylase (Figure 1.3). This enzyme carboxylates glutamic acid (glu) residues on the propeptide to produce gamma-carboxyglutamic acid (gla) residues on functional zymogens (Michal, 1999). In this carboxylation step, vitamin K hydroquinone is used as a cofactor and converted to Vitamin K epoxide (Vermeer and Schurgers, 2000). The importance of the gla residues on coagulation factors II, VII, IX and X, and coagulation proteins C, S, and Z (Mann et al., 1990) is their ability to attract the divalent cation of calcium whose positive charge enables clotting factors to bind temporarily with the polar heads of thrombocyte and endothelial cell membranes (Michal, 1999).

The vitamin K cycle relies upon vitamin K epoxide reductase (VKOR) to convert vitamin K epoxide back to its hydroquinone form (Whitlon et al., 1978; Larson et al., 1981). Warfarin binds to VKOR in the membrane of the hepatic endoplasmic reticulum, disrupting vitamin K cycling between the epoxide and hydroquinone forms (Whitlon et al., 1978; Mackman et al., 2007; Figure 1.3). The enzyme-inhibition complex appears to form when the side-chain at the carbon-3 position on the 4-hydroxycoumarins bind to the disulfide on the VKOR active site and mimics the transition state of water during the production of 2-hydroxyvitamin K (Silverman, 1980; Gebauer, 2007). As a result, less vitamin K hydroquinone is available to gamma-glutamyl carboxylase for the carboxylation of vitamin K-dependant coagulation proteins. The potency and persistence of brodifacoum in comparison to warfarin is likely due to the large lipophilic group attached at brodifacoum's carbon-3 position (Hadler and Shadbolt, 1975). This

side-chain, along with the 4-hydroxycoumarin moiety enables brodifacoum to bind strongly at the VKOR active sites (Gebauer, 2007), blocking what is already a rate limiting step for vitamin K hydroquinone in the gamma-carboxylation of glutamic acid residues (Wallin et al., 2003). It has been proposed that the active site of this enzyme is composed of three segments for binding of the propeptide, glutamic acid residues and vitamin K hydroquinone, and that this results in an induced fit of the vitamin K hydroquinone (Berkner, 2000). This would then cause undercarboxylated proteins, with fewer functional gla residues, to be released by the enzyme and critical levels of functional coagulation factors would not be reached *in vivo*.

It is generally accepted that all vertebrate species have a similarly functioning coagulation system. Structural similarities between prothrombin, coagulation factor II, as well as other coagulation factors (Banfield et al., 1994; Davidson et al., 2003) and VKOR (Rost et al., 2005) are present in many vertebrate species suggesting the coumarin rodenticide mode of action would be highly similar among vertebrate species. There has been some debate on whether avian species possess an intrinsic coagulation pathway; however, evidence suggests the pathway is present but not used as a primary line of defense (Doerr and Hamilton, 1981; Griminger, 1986; Morrisey et al., 2003). In general, differences in metabolism and the quantity of vitamin K in the diet, are likely to impact the susceptibility of each individual and species to the coumarin anticoagulants.

1.2.5 Anticoagulant rodenticide symptomology

After the target rodent consumes a lethal dose of anticoagulant rodenticide bait, its behaviour prior to death may contribute to poisoning of predators and scavengers. Loss of hemostasis causing internal and external bleeding from small wounds leads to death approximately 3 to 10 days after bait consumption (Hadler and Shadbolt, 1975). During the period prior to death, the exposed rodent may continue to feed on bait and accumulate more chemical than is necessary to cause lethality (Cox and Smith, 1992; US EPA, 2004), potentially resulting in high dose to the non-target predator. Specifically, large male rats have been reported to feed in bait stations for up to 2 hours while aggressively defending the bait from smaller rats (Taylor and Thomas, 1989). After anticoagulant exposure, rats have been reported to continue moving throughout their territories while feeding despite leaving a blood trail, and males were found to stagger in open areas in the daylight (Cox and Smith, 1992; Howald, 1997). That behaviour, along with an altered startle response from bolt to freeze, may increase the rodent's vulnerability to predation and the risk of secondary poisoning to predators (Cox and Smith, 1992). Behavioural changes may also increase scavenger exposure since rats have been reported to move out of burrows into open areas to die (Cox and Smith, 1992).

1.3 Risk of secondary rodenticide poisoning

1.3.1 Incidence of non-target poisoning

Although brodifacoum has been used successfully in many settings to

eradicate invasive rats, particularly on islands, there have been numerous reports of non-target species poisoning (Hegdal and Colvin, 1988; Newton et al., 1990; Howald et al., 1999; Eason et al., 2002; Walker et al., 2008). Between 1981 and 2004, more than 300 poisoning incidents of birds and non-target mammals were documented in the United States alone, with 244 of these associated with brodifacoum exposure (US EPA, 2004). Most recently, an attempt at eradicating rats using an aerial baiting campaign on Rat Island in the western Aleutians resulted in no living rats being found, but also resulted in the deaths of 43 Bald Eagles, 213 glaucous-winged gulls and birds of other species (Woods et al., 2009). Monitoring and further research on the effects of rodenticides on that island is required to determine if the eradication was a success and how the non-target species came to accumulate lethal levels.

1.3.2 Potential exposure pathways

Risk of non-target brodifacoum and other SGAR poisoning is through several potential exposure routes including direct consumption of bait and through secondary poisoning associated with consumption of the carcass of the target rodent or the target animal in a weakened state. Insectivores may consume insects which feed directly on bait. Snails, banana slugs and blowfly larvae were reported to contain brodifacoum residues after the Langara Island eradication project and song sparrows containing brodifacoum were thought to have fed on carrion insects (Howald, 1997). Brodifacoum was also found in tissues of birds from a zoo aviary thought to be due to consumption of pavement ants and cockroaches which fed on brodifacoum baits (Godfrey, 1985).

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Secondary poisoning may also occur through predator and scavenger consumption of poisoned rodents (Gray et al., 1992; Gray et al., 1994a; Howald et al., 1999). The common raven (*Corvus corax*) population experienced a mortality rate greater than 50% after feeding on both rat carcasses and directly on bait after the Langara Island eradication program (Howald, 1997). Tertiary poisoning may also occur through predator ingestion of a scavenger. For example, during the eradication on Langara Island, a bald eagle was suspected to have consumed a poisoned raven (Howald et al., 1999).

With a high log K_{ow} of 8.5 (PMRA, 2004), brodifacoum is lipophilic and adsorbs to soil particles minimizing the likelihood of leaching into water (Stevens and Hill, 1979). However, brodifacoum is also susceptible to degradation by soil bacteria which produce non-toxic metabolites (Shirer, 1992). Plants have been shown to not take up significant levels of brodifacoum from the soil (Hendley and McIntosh, 1982) and are an unlikely source of exposure to herbivores.

1.4 Brodifacoum regulation

Brodifacoum was assessed along with several other rodenticides for re-registration by the Pesticide Management Regulatory Agency in Canada and the Environmental Protection Agency in the United States (PMRA, 2004; US EPA, 2004). In both risk assessments a risk quotient (RQ=ppm active ingredient in bait/LC_{50}) was calculated for primary acute and chronic exposure, and mean percent mortality of secondary poisoning studies was used to assess secondary risk. Among the compounds evaluated in both countries, brodifacoum, followed

closely by difethialone, was determined to pose the greatest primary chronic and secondary risk to birds. In Canada, this assessment prompted a regulatory amendment for use and labeling of all products containing brodifacoum to read "For indoor use only" (PMRA, 2006) without similar requirements for difethialone. In the United States, brodifacoum labeling must read "For use in and around agricultural buildings only" and "Do not apply further than 50 feet from buildings" (US EPA, 2008). The complete risk assessment conducted by the US EPA (2004) included recommendations for 'biomarkers' to be developed for exposure of different bird species, residue-monitoring programs to be established where anticoagulants are used in the field, and for reproductive NOAECs (no-observable-adverse-effects-concentrations) to be established for each rodenticide.

1.5 Biomonitoring of anticoagulant exposure

Assessing SGAR exposure in free-living avian populations is most often accomplished by collecting carcasses, conducting necropsies and measuring liver residues (e.g. Albert et al., 2009; Stone et al., 2003). If the bird is found to have high levels of rodenticide particularly in combination with typical anticoagulant poisoning symptoms (described above), it is often included as a rodenticide poisoning incident in risk assessments (e.g. US EPA, 2004). The challenge is how to monitor exposure to these chemicals in non-target birds prior to them causing death. Monitoring of free-living birds to anticoagulant rodenticides should ideally be non-lethal and not cause excessive stress to the

individual or population. It is advantageous to measure coagulation capacity using well developed and standardized clinical test methods from the medical field where anticoagulants have been used therapeutically for over 60 years (Duxbury and Poller, 2001). The veterinary field has adapted those tests for certain mammalian species, however, further modifications, for example to sample collection techniques, are required for avian species to account for their smaller size and vein fragility (Harr and Myers, in press). Another monitoring method which could be used in owls and other pellet-producing species is to measure the rodenticide residues in pellets. Gray et al. (1994b) has shown that 25% of the dose administered to barn owls was eliminated in the pellets. Although this method is non-invasive, it relies upon chemical analysis, an expensive and time-intensive process and it may not be possible to link the pellet with a particular individual.

Anticoagulant rodenticide monitoring may be achieved using a variety of coagulation tests which require careful, clean blood sampling and storage techniques. However, monitoring of some small avian species may not be possible due to the need for clean venipuncture and limitations posed by vein and needle size. Two test methods used clinically to assess anticoagulant exposure are the activated clotting time (ACT) and prothrombin time (PT) tests. The ACT test is simple, can be performed at the time of venipuncture in the field, and assesses completely functional intrinsic and common coagulation pathways (Bateman and Mathews, 1999). The PT test is done in the lab and relies on functional extrinsic and common pathways being activated by tissue factor (FIII)

and phospholipids present in a thromboplastin extract (Doerr et al., 1975). In general, with both assays, the amount of time it takes for a clot to form is recorded and an increase above species baseline levels indicates hemostatic disruption (Bachmann and Sullivan, 1983; Bailey et al., 2005).

1.6 Study species

1.6.1 Natural history of barn owls

Barn owls are a common medium-sized owl found in many parts of the world; the largest subspecies, *Tyto alba pratincola*, is present in North America (Marti, 1992). In BC, this non-migratory species is present throughout the year in the Fraser Valley as far east as Hope, southern Vancouver Island, and a localized population is present in south-central BC (Campbell et al., 1990; Marti, 1992). The total population of barn owls was estimated at 1000 in 1984 (Campbell and Campbell, 1984). Barn owls hunt by flying low, about 1.5 – 4.5 m above ground (Bunn et al., 1982), over open lowland areas including agricultural fields, marshes, grasslands and urban areas (Campbell et al., 1990). Prey includes many small nocturnal mammals such as voles, rats, mice, shrews and occasionally birds, with Townsend's vole (*Microtus townsendii*) comprising 65-83% of the annual diet in southwestern BC (Campbell et al., 1987). Barn owls tend to make sharp changes in direction as they try to locate prey in complete darkness using very sensitive, directional hearing provided by their heart-shaped facial disc (Knudsen, 1981).

Generally monogamous, barn owl pairs often stay together for as long as they live, however new mating pairs are established if one partner disappears (Marti, 1992). Nesting sites include natural tree cavities, barns, attics, steeples, and nest boxes (Marti, 1992) with 94% of nest sites in BC occurring in man-made structures (Campbell et al., 1990). Breeding may occur throughout the year in BC, however, most egg-laying occurs between March and May (Campbell et al., 1990). The female will lay one egg every 2-3 days in a nest of regurgitated pellets, resulting in an average clutch size of 3 - 5 eggs (Marti, 1992; Campbell et al., 1990). Incubation of about 30-34 days (Godfrey, 1986) is performed by the female (570 g) while the male, which weighs on average 100g less than the female, hunts to provide food (Marti, 1992). The first flight of young barn owls occurs at 50-55 days (Bunn et al., 1982), but fledglings tend to return to the nest to roost for several weeks (Lenton, 1984). This nest may also be used during the non-breeding season for roosting (Andrusiak and Cheng, 1997). On average, wild barn owls have an approximate survival of 21 months (Keran, 1981).

Barn owls in BC are currently listed as a species of special concern by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2001), with a reassessment scheduled to occur in April, 2010 (COSEWIC, 2009). The barn owl's status of special concern is due to its small population, and preferred habitat of agricultural fields and pastures which are being lost to agricultural intensification and urban developments (COSEWIC, 2001). The removal of trees and derelict barns reduce potential nest sites. The reduced need for hedgerows

and fallow fields in agriculture decreases habitat for small mammals, and the use of pesticides (including rodenticides) on farmland, together may reduce the quality of foraging habitat (Fraser et al., 1999). An ongoing study by Environment Canada is assessing how changes in land use are affecting barn owls in the Lower Fraser Valley of BC. To date, the data suggests that there is a decline in nest-site occupancy when compared to surveys from 1991-1993 and this is in part due to 35% of these nest sites having been lost (Hindmarch, in prep). Rodenticide residues were also measured in livers from three species of owl in BC and 70% were found to contain at least one anticoagulant rodenticide, with brodifacoum (0.001 to 0.927 ppm) and bromadiolone (0.002 to 1.012 ppm) being most frequently detected (Albert et al., 2009). Although rodenticide poisoning is not a primary cause of death amongst barn owls, a weakened state may contribute to some of the most common known causes of mortality including collision with vehicles, starvation and shooting (Hegdal and Colvin, 1988; Campbell et al., 1990).

1.6.2 Japanese quail – A model laboratory species

Japanese quail (*Coturnix japonica*) are a small (100-160g), rotund, terrestrial bird of the order Galliformes and family Phasianidae, closely related to the chicken. Quail can be raised inexpensively in relatively high numbers and compact spaces. Japanese quail are sexually dimorphic with adult plumage apparent at about six weeks of age and sexual maturity attained at 4-5 weeks of age enabling rapid multi-generational studies (Cheng et al., in press). This species is also very sensitive to photoperiod allowing for manipulation of fertility

and sexual maturity in both sexes. The Universities Federation of Animal Welfare Handbook on the Care and Management of Laboratory Animals includes a thorough chapter describing animal care requirements for Japanese quail including many standard haematological and other baseline data (Cheng et al., in press). A standard test method for sub-acute dietary toxicity has also been developed for the Japanese quail by the American Society for Testing and Materials (ASTM, 1997). Several studies have also successfully used the species as a model for toxicological studies. Elliott et al. (1997) used Japanese quail to examine liver enzyme capacity in response to polychlorinated biphenyl (PCB) exposure considered at that time to be a known PCB-sensitive species for comparison to assess American Kestrel response to identical experimental conditions. Hexanitrohexaazaiso-wurtzitane (CL-20), a propellant under consideration for military has the potential for soil contamination, was assessed using Japanese quail standardized testing methods because of the species' significant dietary exposure to soil through its foraging and behavioral habits (Bardai et al., 2005). In our study, Japanese quail proved to be tolerant of handling and experimental manipulation, including oral dosing and jugular blood sampling, and are therefore a useful species for studying the sub-lethal effects of oral brodifacoum or other anticoagulant exposure through clean venipuncture.

1.7 Thesis objectives

In Chapter 2, I describe a laboratory experiment using captive Japanese quail (*Coturnix japonica*) as an avian model species to assess sub-lethal and lethal effects of anticoagulant exposure. Specifically, I validated two coagulation

tests, prothrombin time (PT) and activated clotting time (ACT), in Japanese quail to determine if oral exposure to the anticoagulant rodenticide brodifacoum can be estimated through measurement of coagulation disruption. The PT test requires clinical laboratory facilities and is more accurate and reliable for assessing the level of exposure, while the ACT test might be applicable to field studies and be of use in assessing wildlife poisoning incidents on site. Due to the delicate nature of coagulation testing, the second aim of this study was to examine the most appropriate method of venipuncture and the effect of storage conditions (e.g. freezing) on prothrombin time to determine appropriate handling of plasma samples for future use in potential monitoring studies. Finally, prothrombin time was measured in plasma collected from free-living barn owls (*Tyto alba*) in the Fraser Valley, BC, to estimate potential anticoagulant rodenticide exposure based on results from the quail brodifacoum exposure study. Chapter 3 discusses the contributions of this study to the current understanding of avian hemostasis, the potential of coagulation tests to be used as a biomonitoring tool and future research recommendations.

1.8 Tables

Table 1.1: Rodenticide usage in British Columbia (2003) and in the United States (1997).

Active Ingredient	Mode of Action[a]	Mg ai/ Kg bait[b]	British Columbia (2003)		United States (1997)		
			Kg ai[c]	Kg bait[d]	Kg ai[e]	Kg bait [d]	OTC Units[e]
Brodifacoum	2C	50	0.42	8,400	200	4,000,000	44,144,456
Bromadiolone	2C	50	0.53	10,600	74	1,480,000	294,706
Diphacinone	I	50	0.15	3,000	275	5,500,000	2,860,419
Chlorophacinone	I	50	0.13	2,600	1,214	24,280	18,360
Difethialone	2C	25	0.081	3,240	U	U	U
Warfarin	1C	250	U	U	U	U	U
Cholecalciferol	D	750	0.080	106.7	U	U	U
Bromethalin	A	100	0.006	60	U	U	U
Strychnine	A	U	47	U	U	U	U
Zinc Phosphide	A	20,000	125	6,250	U	U	U

U=Data unavailable

[a] 1C=First generation coumarin anticoagulant; 2C = Second generation coumarin anticoagulant; I = Indanedione anticoagulant; A = acute rodenticide, D = Sterol causing hypercalcemia (US EPA, 2004).

[b] (US EPA, 2004)

[c] (Environment Canada et al., 2005)

[d] Calculated kilograms of bait based on active ingredient used and average bait concentration.

[e] Total amount of active ingredient (ai) imported or produced in the US including professional applicators, agricultural applications and over-the-counter (OTC) placement units sold to the public (Kaukeinen et al., 2000).

1.9 Figures
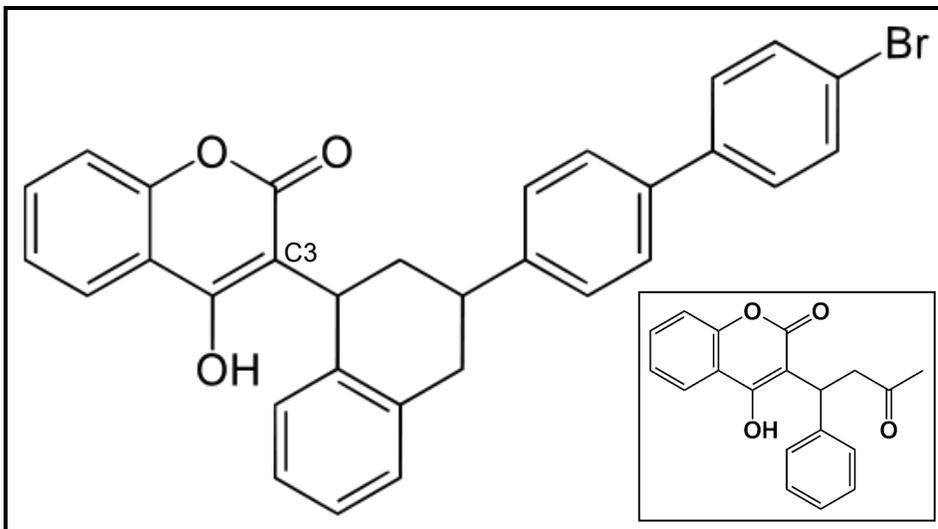
Figure 1.1: Chemical structure of brodifacoum. C3 identifies the 3-carbon position to which the hydrophobic side-chain is attached. Inset: warfarin.
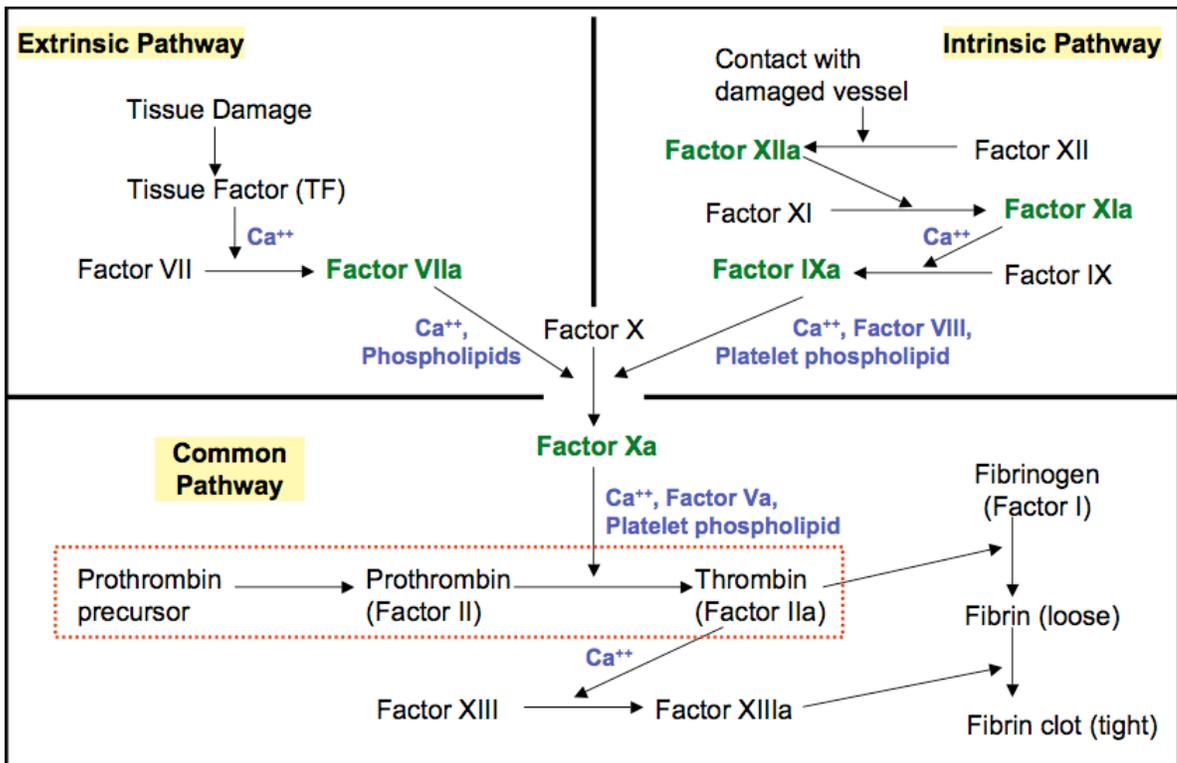
Figure 1.2: Basic coagulation pathways in mammals as designated for diagnostic purposes (not all positive feedback pathways and coagulation inhibitory pathways are shown).
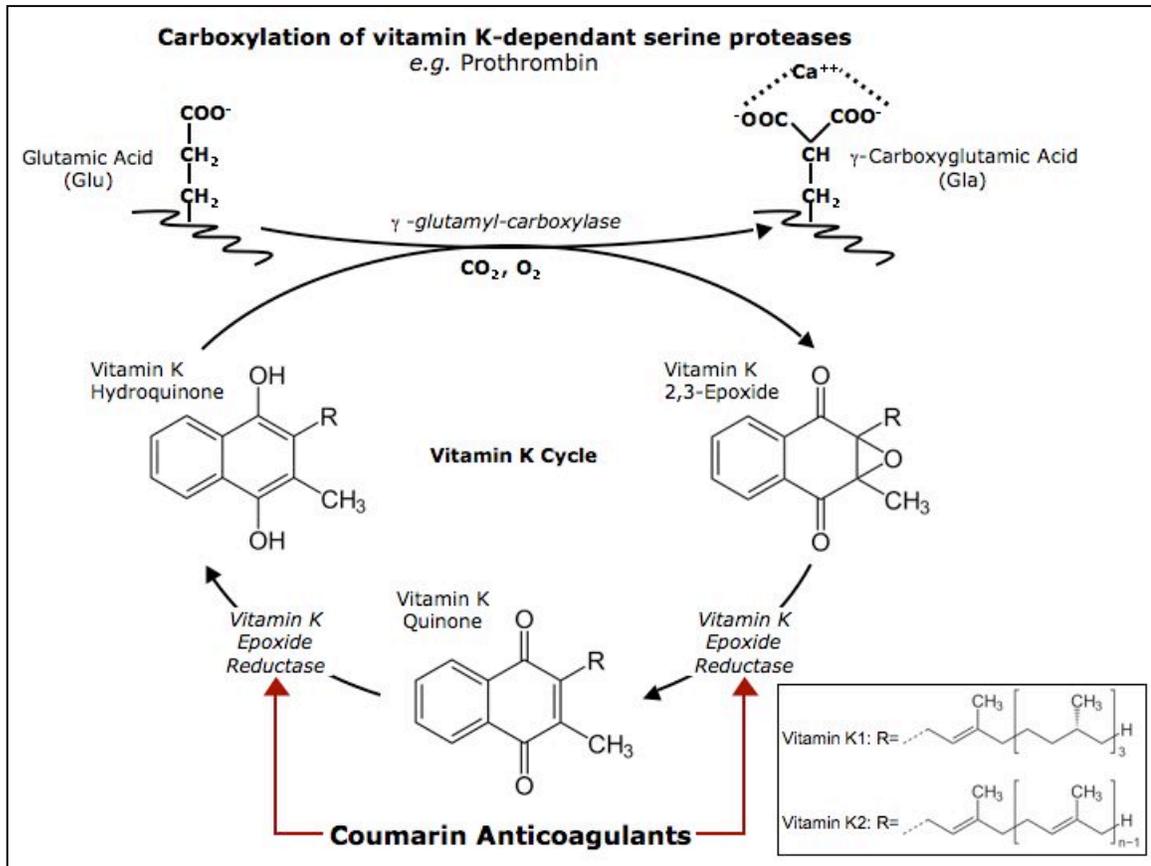
Figure 1.3: Disruption of the vitamin K cycle by coumarin anticoagulants.

CHAPTER 2: THE EFFECT OF BRODIFACOUM ON THE PROTHROMBIN TIME OF JAPANESE QUAIL: A POTENTIAL MODEL FOR ESTIMATING EXPOSURE IN WILD BIRDS

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2.1 Introduction

Second generation anticoagulant rodenticides (SGARs) are effective at controlling invasive rodent populations in agriculture and for wildlife conservation, but they have also been linked to deaths of raptors, including barn owls, through secondary poisoning (Newton et al., 1990; Howald et al., 1999; Albert et al., 2009; Woods et al., 2009). For example, one study in New York, SGARs were detected in livers of 48% of 265 diurnal raptors and owls analyzed, including 81% of 53 great horned owls, 58% of 78 red-tailed hawks, and 45% of 22 Eastern screech-owls (Stone et al., 2003). Hepatic brodifacoum residues were also measured in owl carcasses from the Lower Fraser Valley of British Columbia. In this investigation, 45% of barn owls had been exposed to brodifacoum based on liver levels ranging from 0.01 to 0.47 mg/kg, and up to 0.927 mg/kg in one barred owl (*Strix varia*; Albert et al., 2009). Evidence of non-target species poisoning from SGARs led to a recent re-evaluation and extensive assessment of risks of

SGAR uses in the United states and Canada (PMRA, 2004; US EPA, 2004). That assessment determined that brodifacoum and difethialone posed the greatest hazard of primary and secondary poisoning of birds and non-target mammals for primary and secondary exposure. Since this initial assessment, a 2008 mitigation decision in the United States (US EPA, 2008) limited the availability of baits containing brodifacoum to the public, but did not change how it was used in livestock production or other professional settings. In agriculture, bait stations must be used, but cannot be placed more than 50 feet from buildings. In Canada, brodifacoum end use products are labeled for indoor use only, however labeling currently required for difethialone, which was determined to have very similar risk levels, is "indoors and against the outside walls of buildings" (PMRA, 2006).

Brodifacoum binds strongly to vitamin K epoxide reductase in the endoplasmic reticulum of hepatocytes and blocks the conversion of vitamin K from its epoxide to hydroquinone form (Hadler and Shadbolt, 1975). Vitamin K hydroquinone is required as a cofactor for the γ-carboxylation of glutamic acid residues on several coagulation propeptides (factors II, VII, IX and X; proteins C, S, and Z; Mann et al., 1990). The resulting γ-carboxyglutamic acid (gla) residues are essential for functional clotting activity and brodifacoum exposure therefore, reduces the number of functional clotting factors (Berkner, 2000). When critical threshold levels of these proteins are not reached in the blood, hemostasis is significantly disrupted causing internal hemorrhaging (Griminger, 1986). Brodifacoum is potent, and one feeding can cause death in the target rodent 3-10

days after ingestion (Hadler and Shadbolt, 1975). During the period prior to death, the rodent may exhibit behavioural alterations that potentially increase their susceptibility to predators and scavengers (Cox and Smith, 1990; US EPA, 2004). Rodents may continue to feed upon the bait after a lethal dose of brodifacoum has been ingested (Cox and Smith, 1990; US EPA, 2004), and with a liver elimination half-life of up to 350 days (Batten and Bratt, 1990) a significant portion may accumulate in the liver. In general, mammals appear to be more susceptible to brodifacoum (LD_{50}s < 0.5 mg/kg bw), than avian species (LD_{50}s range from < 1 mg/kg for the pukeko (*Porphyrio porphyrio*) to > 20 mg/kg for the paradise shelduck (*Tadorna variegata*) (Godfrey, 1985; WHO, 1995; Eason et al., 2002)).

Measuring liver residues in individual animals is the most common and accurate method of determining brodifacoum exposure, however, wildlife monitoring of free-living animals should ideally be non-lethal and minimally-invasive, in order to reduce stress on the individual or population. Despite brodifacoum being a persistent rodenticide, it's half-life in serum is only 6.5 days (Bachmann and Sullivan, 1983), thus there is limited utility in measuring serum or plasma residue concentrations as an index of exposure in free-living individuals. Gray et al. (1994b) found that brodifacoum residues can be measured in the pellets of barn owls which contain approximately 25% of the oral dose. Although this monitoring method is non-invasive, it also relies upon expensive and time-intensive chemical analysis, and it is not always possible to determine which bird produced the contaminated pellet. Relative to chemical analysis in liver, plasma,

or pellet, coagulation disruption tests are quite rapid and inexpensive, requiring few supplies other than a coagulation analyser. Although coagulation tests may be influenced by venipuncture technique or sample handling and storage (Harr and Myers, In press), standardized test methods have been developed in mammalian species and can provide a rapid indication of coagulation disruption (Duxbury and Poller, 2001).

The barn owl (*Tyto alba*) in the Lower Fraser Valley of British Columbia, is listed as a species of special concern primarily due to their barn-nesting and field-foraging habits which conflict with increasingly industrialized agricultural practices, road numbers, and pesticides including rodenticides (Fraser et al., 1999). It has been shown that brodifacoum has greater secondary toxicity to barn owls compared to bromadiolone (Mendenhall and Pank, 1980) and difenacoum (Newton et al., 1990). Brodifacoum is found in livers of deceased owls at levels ranging from 0.001 to 0.97 mg/kg (Albert et al., 2009; Stone et al., 2003). Although a threshold level for brodifacoum toxicity has been suggested at a liver residue of 0.7 mg/kg (Gray et al., 1994a), there is a lot of uncertainty surrounding what minimum concentration causes toxicity, and it is essential to monitor the species potentially at risk of exposure.

The first objective of this study was to determine if brodifacoum exposure can be estimated using either of two coagulation tests: the prothrombin time (PT) and activated clotting time tests, following oral exposure to brodifacoum in the model laboratory species Japanese quail (*Coturnix japonica*). Secondly, the effect of blood sampling technique and sample storage conditions were assessed

on the use of coagulation tests. Thirdly, prothrombin time was measured in plasma obtained from free-living barn owls in the Fraser Valley, B.C., to estimate potential anticoagulant rodenticide exposure based on results from the brodifacoum PT validation study.

2.2 Materials and methods

2.2.1 Study species and animal housing

Japanese quail (*Coturnix japonica*) were obtained from the Agassiz Poultry Research Centre (Agriculture and Agri-Food Canada, Agassiz, BC, Canada) at 5 weeks of age and were acclimated for 17 weeks at the University of British Columbia (UBC) Animal Care Centre. Upon arrival, birds were each banded with plastic split-ring leg bands (AC Hughes, UK) and were randomly assigned to one of 5 large plastic bins (0.91 m W x 1.6 m L x 61 m H) containing wood shavings to allow for dust bathing and pecking. Birds were visually, but not acoustically, isolated from other groups. Temperature was 21±1°C and day length was limited to 8 hours per day. Quail were provided with water and food *ad libitum*, containing 26% protein and 1 mg of vitamin K per kg feed. Animal husbandry and experimentation was conducted under UBC and Simon Fraser University animal care permits following guidelines of the Canadian Council on Animal Care.

2.2.2 Experimental design

Adult quail were divided into 5 treatment groups (n=24: 12 male, 12 female). Exposure doses were determined following two preliminary trials using

single brodifacoum (98.9% purity, Syngenta Inc.) doses of 2.3 and 3.75 mg/kg. At these doses, no morbidity or mortality occurred, and at necropsy only one bird (3.75 mg/kg) had evidence of internal haemorrhaging. All brodifacoum dosing solution concentrations were determined using LC-MS (Pacific Environment Science Centre, Environment Canada, North Vancouver, B.C.). The brodifacoum solutions in corn oil were dissolved in hexane and liquid/liquid extracted into acetonitrile. The extracts were then run on an LC-MS (1100 Series, Agilent Technologies) using negative electrospray ionization in selected ion monitoring mode. Final doses were chosen to be sublethal, and were determined by this analysis to be 0, 0.8, 1.4, 1.9, and 2.5 mg/kg.

Each quail was dosed by gavage with 0.5 ml corn oil per 100 g body weight to achieve the above doses. Body mass (± 0.1 g) was recorded immediately prior to dosing (Day 0) and prior to blood sampling and euthanasia. Liver somatic indices (LSI=liver weight/ body weight) were calculated based on body weight prior to sampling. During the exposure period, birds were monitored for signs of distress, or external haemorrhage and euthanized if these symptoms were observed to be severe. After 1, 3, 5 and 7 days of exposure, 6 birds from each dosing group were blood sampled by clean venipuncture as described below and immediately euthanized with isoflurane (Carpenter, 2000). Necropsy was performed to record evidence of internal haemorrhaging, and livers were removed for residue analysis (chemistry data pending).

2.2.3 Blood collection and processing

Blood collection for the prothrombin time (PT) and activated clotting time (ACT) assays were by clean venipuncture using a 23 to 27 gauge needle in the right jugular vein in order to minimize tissue factor contamination of the sample. Immediately after venipuncture, 0.5 ml of blood was added to the ACT tube, and 3.8% (w/v) sodium citrate (SC) in a 1:9 ratio (SC: blood) for the PT test. The prothrombin time sample was inverted several times to mix, centrifuged for 3 min of 13,400 g, and plasma was transferred to a cryogenic vial, frozen in liquid nitrogen and transferred to a -80°C freezer until assayed. Additional heparinized microcapillary tubes of blood were collected to measure hemoglobin and hematocrit.

2.2.4 Activated clotting time

The ACT tube contained 3 mg of diatomaceous earth and was incubated at 37°C prior to the addition of 0.5 ml blood (Bateman and Mathews, 1999). The tube was inverted several times to mix, incubated for 1 min at 37°C and examined for microclot formation. The tube was checked frequently and returned to 37°C between observations until a microclot was recorded. All ACT test observations were performed by the same individual.

2.2.5 Prothrombin time

The thromboplastin extract (containing tissue factor) was prepared by incubating 50 mg of acetone-dried chicken brain powder (Lot #16529; Innovative Research, Michigan, USA) in 2.5 ml of 0.025M $CaCl_2$ at 42°C for 15 minutes,

vortexing every 3 min (Doerr et al., 1975). The suspension was then centrifuged at 3170 rpm for 10 min, the supernatant was stored on ice, and an equal volume of 0.025M calcium chloride was added. All prothrombin times were measured with thromboplastin extracted within 4 h.

Prothrombin time was measured using the STart 4® coagulation analyzer (Diagnostic Stage) at Idexx Reference Laboratories, Ltd. (Delta, BC, Canada). Frozen plasma samples were first thawed in a 37°C waterbath for 3 min. Plasma and thromboplastin were incubated at 37°C for an additional 3 min in the STart 4 analyzer prior to adding 100 μl of thromboplastin extract to activate 50 μl of plasma. When a clot is formed, the STart 4® detects an increase in viscosity is detected and the prothrombin time is reported.

To determine the effect of sample storage on prothrombin time, blood was collected from an additional 14 untreated Japanese quail, plasma was pooled and divided into 0.6 ml aliquots. The prothrombin time of one fresh aliquot was measured and all remaining aliquots were immediately frozen in liquid nitrogen and subsequently stored at -80°C. Prothrombin time was measured in these plasma pools after freezing for 3, 5, 7, 9 and 28 days, then every 4 weeks up to 7 months. To determine the effect of reduced clotting protein concentration, one aliquot of the above plasma pool was diluted with phosphate buffer (pH 7.4, 8.3 mM) and PTs were measured at 10, 20, 40, 60, 80, and 100% plasma (Bailey et al., 2005).

Prothrombin times were also measured in normal and abnormal mammalian plasma standards (Pacific Hemostasis® Coagulation Controls Level

1 and 2) using chicken thromboplastin. The intent of these PTs was to establish a reference range for a standardized plasma to control for the inter-day variation associated with thromboplastin extraction if individual barn owl samples are later measured for monitoring programs.

2.2.6 Hemoglobin and hematocrit

Hemoglobin (Hb; g/dl whole blood) was measured using the cyanmethemoglobin method of Drabkin and Austin (1935) modified for use with a microplate spectrophotometer (BioTek Powerwave 340, BioTek Instruments, Ltd., Winooski, VT, USA) using 5 μl of whole blood and 1.25 ml of Drabkin's reagent (D5941, Sigma Aldrich Canada Inc.) with absorbance read at 540 nm. Standard curves were used to calculate Hb concentration using a cyanmethemoglobin standard (StanBio Laboratory, Texas, USA).   Hematocrit (Hct; % packed red blood cells) was measured in heparized microcapillary tubes following centrifugation of whole blood for 3 min at 13,400 g (Campbell and Ellis, 2007).

2.2.7 Barn owl sampling

Blood samples were collected from 46 barn owl chicks with nest sites in the Lower Fraser Valley between April and October 2008. Weight, tarsus and wing lengths were recorded for each bird (Hindmarch, in prep). Blood was collected by clean venipuncture from the brachial vein with a 26 to 27 G syringe rinsed with 3.8% (w/v) SC. Sodium citrate was added immediately in a ratio of 1:9 (SC:blood) and inverted to mix. Blood was centrifuged within 4 h of sampling

and plasma samples were frozen in liquid nitrogen until stored at -80°C. Prothrombin times were measured as described for Japanese quail.

2.2.8 Statistical analysis

All statistical analysis were carried out using JMP software version 7.0.2 (SAS Institute, 2007). To confirm that data met the assumptions for a parametric ANOVA, it was first assessed for equal variance and normality using the Shapiro-Wilk test. As the data from the coagulation tests and liver somatic index were found to be not normally distributed, it was subjected to the non-parametric Kruskal-Wallis test to determine the presence of a treatment effect both between groups and over time. Differences between treatment groups over time were determined by post-hoc Wilcoxon tests with p-values adjusted using the Bonferroni method to account for multiple comparison effects. PT assay validations for freezing and dilution studies were analyzed using a bivariate linear and nonlinear regression models, respectively. Hemoglobin, hematocrit and weight data, which met the assumptions for parametric analysis, were analyzed by two-way ANOVA. This resulted in a significant dose*time interaction and further one-way ANOVAs for each dose group and time period were conducted along with the conservative Tukey Kramer's HSD post-test (α=0.05) to determine the location of the differences between groups. Barn owl coagulation data from the field study described above was analyzed using standard parametric statistics to report baseline coagulation values for this species, and differences with quail groups were determined as described above for quail PT analysis.

2.3 Results

2.3.1 Body condition and mortalities

There was no significant change in body weight (control mean 128.7 g) or liver somatic index (LSI=100% x liver weight/body weight; control mean 2.2%) among dose groups or over time (Appendix A). During the seven days post-exposure a mortality rate of 2 (8.3%), 4 (16.7%), 3 (15%), and 5 (20.8%) quail either died, or were euthanized due to severe haemorrhage, at the 0.8, 1.4, 1.9[1] and 2.5 mg/kg bw dose groups, respectively, with the first death occurring two days after exposure (Table 2.1). External bleeding occurred on the lower back at the site of new feather growth in 4 females in the 2.5 mg/kg bw dose group, in 1 female and 1 male in the 1.9 mg/kg bw dose group, and 1 male in the 1.4 mg/kg bw dose group. External examination of some individuals at the highest dose groups also revealed pale extremities or bluish tarsometatarsus. Necropsies revealed subdermal haemorrhage of the cranium, legs and wings, bloody vitellogenic follicles, greyish testis, haemorrhage into the abdominal and orbital cavities, and blood in the gastrointestinal system, including crop, gizzard and possibly intestines. Some of these symptoms were experienced at all dose groups including 0.8 mg/kg bw, however no haemorrhages or bruising occurred in any of the control birds (see Appendix B for details).

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[1] Note: Due to error on sampling day 1, four extra birds were euthanized and this percentage is out of 20 birds total, rather than 24 as with the other dose groups.

2.3.2 Activated clotting time

There was no significant change in the activated clotting time (ACT) between control group birds sampled on days 1 ,3 ,5 and 7 of the study (c^2(3, n=20)=2.16, p=0.540). In contrast, there was a significant positive effect of brodifacoum dose on ACT 1, 3 and 7 days post-exposure (c^2(4, n=24)=14.49, p=0.006; c^2(4, n=25)=12.49, p=0.014; c^2(2, n=10)=6.95, p=0.031) and 5 days post-exposure showed a similar yet non-significant increase (c^2(4, n=21)=9.11, p=0.059), i.e. ACT was longer with brodifacoum dosing (Figure 2.3). Specifically, significant increases occurred 3 and 7 days post-exposure in the 0.8 mg/kg dose group (c^2(1, n=12)=8.31, p=0.004; c^2(1, n=9)=6.00, p=0.029), 1 day post-exposure at 1.9 mg/kg (c^2(1, n=10)=6.81, p=0.036), and 3 days post-exposure at 2.5 mg/kg dose group (c^2(1, n=11)=7.50, p=0.025) in comparison to control groups of the same day. There was no significant difference in the ACT over time within the 1.4 and 1.9 mg/kg dose groups when compared with birds sampled on day 1 (c^2(3, n=17)=0.98, p=0.807 and c^2(2, n=11)=2.06, p=0.357, respectively). In contrast, there was a significant dose effect over time within the 0.8 and 2.5 mg/kg dose groups (c^2(3, n=18)=11.93, p=0.008; c^2(2, n=14)=9.93, p=0.007). In the 0.8 mg/kg dose group, pairwise Wilcoxon tests and Bonferroni p-value adjustments eliminated the specific significance increases in ACT 3 and 5 days post-exposure (c^2(1, n=9)=4.27, p=0.12; c^2(1, n=8)=5.00, p=0.076) when compared to the ACT measured 1 day post-exposure. In the 2.5 mg/kg dose group, the ACT measured 3 and 5 days post-exposure increased significantly from that measured 1 day post-exposure (c^2(1, n=10)=6.82, p=0.018; c^2(1, n=9)=6.00, p=0.029).

2.3.3 Prothrombin time

Internal laboratory standards for PT samples of wild birds included normal and abnormal commercial control plasma (Pacific Hemostasis) which were activated using chicken thromboplastin and produced mean prothrombin times of 107.1 s and 182.3 s, respectively (see Table 2.2). The intra-day coefficient of variation for monthly prothrombin time measurements of the frozen (-80°C) plasma pool aliquots was less than 8%. For aliquots frozen up to 7 months, the mean PT decreased over time at a rate of 0.0078 s/day (2.8 s/year; Figure 2.1) but that change was not statistically significant ($F_{(1,11)}=4.83$, p=0.053). The mean PTs for those monthly samples ranged from 10.6 to 13.4 s (mean = 11.9 s, 95% CI 11.3 – 12.6 s). That range was similar to the mean of the individual control quail (Table 2.2) which did not change significantly over the 7 days after receiving the corn oil vehicle. A 90% dilution of this same plasma pool resulted in a 220% increase in PT, while a 60% dilution resulted in only a 22% increase (Figure 2.2).

Inter-individual variation in prothrombin time increased with brodifacoum exposure as shown by comparing the variance of the control group (SEM=0.3 s) with that of each treated group (SEM=1.4 s to 169.9 s). All doses of brodifacoum tested caused a significant increase in PTs compared to same-day controls, 1 and 3 days post-exposure (p<0.05 for all groups; Figure 2.3). A significant time effect occurred in all dose groups at 3 and 5 days post-exposure when PTs were compared with those measured 1 day post-exposure (p<0.05 for all groups). There was also a significant dose effect in PT value 5 days post-exposure (c^2(4,

n=26)=19.63, p<0.001), however, pairwise Wilcoxon tests and Bonferroni p-value adjustments resulted in only the 0.8 and 1.4 mg/kg dose groups differing from controls (c^2(1, n=10)=6.55, p=0.042 for both dose groups); although changes in PT in the 1.9 and 2.5 mg/kg dose groups were close to significant (c^2(1, n=9)=6.00, p=0.057 for both dose groups). Similarly, there was a significant dose effect 7 days post-exposure (c^2(2, n=12)=8.39, p=0.015), but after Bonferroni adjustments, only the 0.8 mg/kg dose group was significantly higher than controls (c^2(1, n=10)=6.59, p=0.021), while the 1.4 mg/kg bw dose group was not (c^2(1, n=8)=4.05, p < 0.088).

2.3.4 Hemoglobin

Hemoglobin concentration varied significantly with dose (F(2,81)=11.31, p<0.0001) and time (F(2, 81)=27.39, p<0.0001) but the interaction was also highly significant (F(10,81)=3.01, p=0.0028), i.e. the pattern of hemoglobin change over time varied with dose (Figure 2.5). There were no significant differences in hemoglobin with time within the control or 0.8 mg/kg dose groups after any exposure period throughout the study (F(3,23)=1.54, p=0.2359; F(3,21)=1.16, p=0.352), or between dose groups 1 day post-exposure (F(4,29)=0.4258, p=0.7885). In contrast, there were significant decreases in hemoglobin over time within the 1.9 and 2.5 mg/kg dose groups on days 3 and 5, and the 1.4 mg/kg dose group on days 5 and 7 when compared to same-dose hemoglobin on day 1 (Tukey HSD, α=0.05). Significant decreases in mean hemoglobin concentrations also occurred due to brodifacoum exposure when compared to same-day controls 3 days post-exposure at the 1.9 mg/kg dose

groups, 5 days post-exposure in the 2.5 mg/kg dose groups, and 7 days post-exposure in the 1.4 mg/kg dose group (Tukey HSD, α=0.05).

2.3.5 Hematocrit

Hemoglobin concentration varied significantly with dose ($F_{(2,82)}$=11.61, p<0.0001) and time ($F_{(2,82)}$=19.69, p< 0.0001) but the interaction was also highly significant ($F_{(10,82)}$=3.39, p<0.001; Figure 2.6). There were no significant differences in hematocrit with time within the control or 0.8 mg/kg dose groups after any exposure period throughout the study ($F_{(3,23)}$=1.77, p=0.185; $F_{(3,21)}$=0.66, p=0.584), or between dose groups 1 day post-exposure ($F_{(4,28)}$=0.49, p=0.745). In contrast, significant decreases in mean hematocrit due to brodifacoum exposure when compared to same-day controls occurred 5 days post-exposure in the 1.4, 1.9, and 2.5 mg/kg dose groups, and 7 days post-exposure in the 1.4 mg/kg dose group (Tukey HSD α=0.05). There were also significant decreases in hematocrit over time within the 1.9 and 2.5 mg/kg dose groups on days 3 and 5 post-exposure, and within the 1.4 mg/kg dose group on days 5 and 7 when compared to same-dose hematocrit on day 1 (Tukey HSD, α=0.05).

2.3.6 Barn owl prothrombin times

The mean prothrombin time of barn owl (BNOW) chicks aged 40-55 days and sampled directly from nest sites was 21.4 s (Table 2.2). Barn owl PT times were significantly lower than PT values for quail in the two lowest doses, 0.8 and 1.4 mg/kg 1 day post-exposure (c^2(1,n=52)=15.40, p<0.0005; c^2(1,n=52)=8.04,

p=0.023) and 3 days post-exposure (c^2(1,n=52)=15.63, p<0.0005 for both doses). However, the mean barn owl PT is significantly higher compared to undosed quail (c^2(1,67)=39.68, p<0.0005). One barn owl chick delivered to a local wildlife rehabilitation centre had a PT of 101.6 s which was in the range of PTs measured in the quail at the 0.8 dose group 3 days after dosing, and at the 1.4 to 2.5 mg/kg dose groups 3 and 5 days after dosing (Figure 2.7).

2.4 Discussion

Our study clearly shows that brodifacoum is a very potent disruptor of coagulation in Japanese quail. All brodifacoum doses greater than or equal to 0.8 mg/kg significantly increased PT even 1 day after treatment but at 1.4 to 2.5 mg/kg there was a strong dose- and time-dependent effect of brodifacoum with PT times averaging 250-400 seconds five days after treatment (compared to a PT time of 13.2 seconds in control birds). At these higher brodifacoum doses there were marked decreases in hematocrit and haemoglobin with evidence of extensive haemorrhaging in post-mortem necropsy. In contrast, at the lowest brodifacoum dose which also resulted in prolonged PT, there were no significant changes in hematocrit or haemoglobin relative to controls, suggesting a more moderate and recoverable physiological effect. The PT measured in aliquots of a quail plasma pool was not significantly decreased over the 7 months of storage at -80°C despite a decreasing trend of 0.0078 s/day. The ACT assay also detected significant increases in coagulation time relative to controls although the dose- and time-dependent pattern seen with the PT assay were not as clearly evident, i.e. the ACT assay estimated more variable coagulation times.

Prothrombin times measured in plasma from free-living barn owls were similar to, though slightly higher than, those in control quail but were much lower than the PT times seen in brodifacoum exposed quail even at the lowest dose level. Although an absolute comparison cannot be made between barn owl and quail PTs, we found little evidence of significant brodifacoum exposure in free-living barn owls, with the exception of one individual (PT=101.6 s).

Brodifacoum disrupts coagulation in Japanese quail as well as numerous other species (Bachmann and Sullivan, 1983; Breckenridge et al., 1985; Mosterd and Thijssen, 1991; Howald, 1997; Bailey et al., 2005). Increases in PT specifically have been reported in response to the anticoagulants diphacinone in the crow (*Corvus corax*) (Massey et al., 1997) and golden eagle (*Aquila chrysaetos*) (Savarie et al., 1979), and warfarin in the chicken (*Gallus gallus domesticus*) (Stopforth, 1970), however, few studies have examined the sub-lethal effect of brodifacoum on PT in avian species (Bailey et al., 2005; Howald, 1997). Clinically, when PT is increased by 25% or more above high normal values, it is considered suggestive of anticoagulant exposure (Shlosberg and Booth, 2006). In our study, a 25% increase in quail PT would be equivalent to approximately 19.3 s which was exceeded in all dose groups as early as one day after exposure. Bailey et al. (2005) found that when chickens were dosed with brodifacoum at 0.75 mg/kg, PT was maximally prolonged seven days after exposure resulting in only 6.1% of normal clotting activity, and PT recovery occurred 28 days after exposure. At a comparable dose in our study of 0.8 mg/kg, PT was maximally disrupted three days after exposure with clotting ability

reduced to only 4.5% of initial values (89.3 s determined from Figure 2.2). Seven days post-exposure, the average PT in this dose group (41.4 s) appeared to begin recovering, though this corresponds to only 8.2% of control values. In contrast, the 1.4 mg/kg dose group exhibited maximal coagulation disruption five days after exposure with only 3.2% (260.0 s) of plasma clotting ability remaining. Peak PT disruption time appears to be inversely dose-dependant with the lowest dose group reaching this peak earlier (by day 3) compared to higher doses which appeared maximally disrupted by day 5. However, without dose groups for day 7 at the two highest doses, this trend requires confirmation. The dose-dependence of peak PT disruption time may be in part due to enterohepatic cycling of brodifacoum resulting in a slow clearance rate from the liver as was suggested by Bachman and Sullivan (1983) when intestinal levels increased from 24 to 72 hours in a similar study with a 0.2 mg/kg dose of brodifacoum administered to rats. As brodifacoum is metabolized in the liver to be eliminated into bile, it would also be continually removed from the blood, maintaining liver saturation levels until the concentration in the blood is reduced. Brodifacoum doses of 1.4 mg/kg and above also resulted in a few birds with extremely long PTs (> 800 s) suggesting that the concentration of circulating clotting factors was under the threshold level for those individuals. The typical threshold behaviour associated with a steep dose-response curve and low therapeutic index (Wadelius and Pirmohamed, 2007) is illustrated. Similarly, when plasma is diluted there is no marked increase in PT until the concentration of coagulation factors is reduced to approximately 10%, but a pronounced increase in PT occurs when the

concentration of clotting factors decreases below this threshold (Figure 2.2). It should be noted that the threshold concentration of functional coagulation factors is specific for each individual.

Haemoglobin and hematocrit values in control birds remained in the normal range (Coenen et al., 1994; Cheng et al., in press) throughout this entire study with no significant change over time. In the 0.8 mg/kg dose group, hematocrit and haemoglobin values were not different from those in same-day control groups, even though these birds had significantly prolonged PTs. In contrast, the 1.4 mg/kg dose group experienced significant reductions in both hemoglobin concentration and hematocrit, from 12.8 g/dl and 40.7% to 3.8 g/dl and 15.2%, respectively, seven days post-exposure. Reduced hematocrit has also been reported after brodifacoum poisoning incidences in the Red-tailed hawk (*Buteo jamaicensis*) and White-winged wood duck (*Cairina scutulata*) of 9% and 16%, respectively (James et al., 1998; Murray and Tseng, 2008). These hematocrit values are indicative of anaemia (generally hematocrit < 35%) (Campbell and Ellis, 2007) and were likely caused by acute vascular blood loss internally or externally, which is supported by necropsy findings. The severe decrease in both hematocrit and hemoglobin seven days post exposure in the 1.4 mg/kg dose group was concurrent with PT values appearing to return towards control values. After the initial rapid phase of hepatic elimination and metabolic clearance below liver saturation levels, it is possible that concentrations of coagulation factors began to increase towards threshold by day 7. However, the concentration of functional clotting factors is still likely less than

10% of control levels, even with PT decreasing towards control levels (Figure 2.2).

Avian response to anticoagulant rodenticides reported in the literature has been variable and mortalities do not always follow a dose-response relationship. For example in one study, after individuals from several avian species were orally dosed with brodifacoum, some birds died at a lower dose but not at a substantially higher dose (Godfrey, 1986). Similarly, brodifacoum administered to rats and fed to different species of owls at comparable intake rates, resulted in different toxic effects that could not be explained from a dose-response relationship (Mendenhall and Pank, 1980). As suggested by some mammalian studies, stress, exercise and diets high in protein may increase or decrease an animal's susceptibility to anticoagulants (Colvin and Wang, 1974; Laliberte et al., 1976; Barber and Colvin, 1980). Finally, aging may increase endogenous procoagulant proteins and decrease anticoagulant proteins (Kurachi et al., 2008), which would increase the tendency for thrombosis and the tolerance of anticoagulants.

Laboratory studies examining the effect of anticoagulants in avian species may require sample sizes larger than is customary due to inter-individual variability caused by minor bruising and wounds from increased interactions, mating and escape attempts that occur when animals live in close proximity. However, it is difficult to spot a moribund bird since symptoms of poisoning may not become apparent until less than 24 hours prior to death, as was found in this study and in owls exposed to six anticoagulant rodenticides (Mendenhall and

Pank, 1980). In this study, the ratio of males to females was higher than recommended (Cheng et al., In press) resulting in increased mating, as well as fighting between males, both of which may have increased the incidence of superficial injuries. Bruising of the tarsus, as well as bleeding into the orbital cavity at the 0.8 mg/kg dose, was observed in Japanese quail likely as a result of minor injury. Bleeding from minor wounds is also a common symptom of moribund free-living birds poisoned with anticoagulant rodenticides and emphasizes the severity of these minor injuries with low-level exposure (Stone et al., 2003; Murray and Tseng, 2008).

We found no significant effect on PT after avian plasma storage at -80°C for up to 7 months. There was a small non-significant decrease in PT of 0.0078 s/day but this did not change the clinical interpretation of the test. Storage conditions of plasma prior to PT analysis is a consideration in the medical and veterinary fields, since samples must be transported to the lab for analysis, sometimes from remote locations, as in the case of biomonitoring. Investigations of the effect of storage conditions on human plasma at room temperature, 4°C, or freezing temperatures have reported conflicting results (Table 2.4). Storage of plasma at room temperature did not significantly alter the PT if measured within 24 hours (Heil et al., 1998; Rao et al., 2000). Storage of plasma at 4-6°C has also produced quite variable results in PT. Rao et al. (2000) found that storage for 24 hours significantly increased the PT, although the increase was not sufficient to alter the clinical interpretation of the results. Another study found that during a 24 hour time period the PT decreased by more than 10% (Heil et

al., 1998). Freezing of plasma samples at -20°C caused statistically significant (but clinically insignificant) prolongation of PT values (Rao et al., 2000) while another study stored samples at -24°C resulting in a decrease of less than 5% after 6 months and less than 10% after 12 months (Woodhams et al., 2001). Storage of plasma at -74°C has been shown to result in PT values with less than 5% variability after 12 months and less than 10% after 2 years (Woodhams et al., 2001), resulting in the practice adopted for this study of archiving plasma samples at -80°C during the field season. Avian plasma samples may also cause variations in PT in response to freeze-thaw cycles. One study determined that chicken plasma could be stored at 4°C without significant changes (Timms, 1977). However, at -20°C the PT was significantly prolonged, and the authors suggested that this appeared to be due to the free-thaw event, rather than the length of time the sample was frozen (Timms, 1977). Chicken plasma was also frozen for 3 months resulting in PTs very similar to those prior to freezing (9.0-15.9s) (Stopforth, 1970).

Although the barn owl blood samples were centrifuged up to a maximum of 4 hours after venipuncture potentially contributing to the PT being slightly higher than that of quail, centrifugation did occur within a time period previously shown to result in stable in PTs. Clinical interpretation of the PT was not affected when whole blood from patients taking anti-vitamin K therapy was stored for up to 6 hours at 4°C and at room temperature prior to centrifugation, possibly due to the buffering ability of hemoglobin (Salvagno et al., 2008). However, storage of whole blood at 4°C for 24 hours significantly prolonged the PT by about 2%

(Salvagno et al., 2008), possibly due to the deterioration of labile factors V and VIII caused by an increase in pH (Thomson, 1984).

In addition to blood or plasma storage conditions, inter-batch variation of thromboplastin extract and its storage conditions may also affect the PT. In contrast to humans and common mammalian pets, there are no standardized techniques or reagents for measuring avian PT. There is little evidence that use of non-homologous (chicken) thromboplastin increases estimated PT times in birds substantially compared with use of homologous thromboplastin (means 12 vs 15 sec; see Table 2.3, excluding data for ostrich). In contrast use of mammalian thromboplastin in avian studies markedly increases estimates of PT time (42 - 170 sec). This variability is most likely due to minor enzyme and clotting factor differences between species. Although different aliquots of thromboplastin may have a significant effect on PT (Morrisey et al., 2003), the present study found that the variation was usually insufficient to alter the clinical interpretation of the results. Stability of thromboplastin is variable and some studies have shown that it must be stored as a dried powder in the freezer, while others report it can be stored as extract at 4°C for 8 months, and at room temperature for 4 hours (Doerr et al., 1975; Timms, 1977). In our study, thromboplastin powder was stored at -20°C and the extract was kept on ice and used within 4 hours to minimize potential variability.

We found that ACT could be measured in Japanese quail, with lower variability in untreated birds than in anticoagulant-exposed birds. We examined the ACT test primarily due to its simplicity and low resource requirement, but also

as a potential screening test which could be used in the field or when injured or debilitated birds are brought into wildlife rehabilitation centres. This assay is intended to assess the intrinsic pathway of coagulation by using diatomaceous earth (DE) as a charged surface to activate the Hageman factor (FXII), initiating the coagulation cascade. Since anticoagulant rodenticides disrupt the production of any vitamin k-dependant clotting factors, including two involved in the intrinsic coagulation pathway, it was expected that the ACT would be prolonged with brodifacoum exposure. In mammals, the ACT is commonly used in veterinary medicine as a screening test when no laboratory is available to assess dysfunction of the intrinsic clotting cascade (Bateman and Mathews, 1999) and has been used to detect warfarin exposure in dogs (Middleton and Watson, 1978). The intrinsic pathway was previously believed to be absent or of limited importance in avian species (Bigland and Triantaphyllopoulos, 1961; Bigland, 1964; Stopforth, 1970), however, recent literature shows general agreement that the contact activation system is likely present, but plays a secondary role in maintaining hemostasis (Doerr et al., 1976; Harr and Myers, In press). We found that the ACT test provided consistent results when birds were not exposed to brodifacoum, but provided false-negative results in some exposed birds (75% sensitivity, 100% specificity)[2] and thus is determined to be a less reliable indicator of anticoagulant rodenticide exposure compared to the PT test (99% sensitivity, 100% specificity). It is recommended that in order to determine the true reliability of the ACT test, a focused study should be conducted to provide

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[2] Sensitivity = # true positive test results / (# true positive test results + # false negative test results)
  Specificity = # true negative test results / (# true negative test results + # false positive test results)

ample blood by clean venipuncture to perform the test with intra-individual replicates and large sample sizes.

Barn owls are an excellent biomonitoring species due to their high trophic position, wide distribution, non-migratory nature, high reproductive rate, relatively easy capture and handling, well studied biology, and sensitivity to a range of contaminants (Sheffield, 1997). Barn owls hunt for small mammalian prey while flying low and at high speeds near urban environments, a strategy which is likely to result in occasional injury and potential exposure to poisoned prey animals.

An absolute comparison cannot be made between barn owl and quail PTs. However, in the absence of a 'true' PT baseline for barn owls (from confirmed unexposed animals) estimations can be made based on the high PTs and inter-individual variation of exposed quail. Our mean barn owl PT was 21.4 s (Table 2.2; n=47) which was significantly higher than the value obtained for control quail (13.2 s), but that PT is also significantly lower than the PT that was measured in quail up to 7 days after brodifacoum exposure at the lowest dose of 0.8 mg/kg. This difference between species is likely due to inter-species variation; however, if barn owl PTs were directly compared to the PT of control quail, 91% of birds would have a PT 25% higher than the high control quail value (higher than 19.3 s). Inter-individual variability for the barn owl PTs was only 14.8% compared to 10.5% for the control quail, which may be expected when laboratory animal variation is compared to that in free-living birds. Furthermore, in this study we found that inter-individual variation increased substantially with brodifacoum exposure. Relatively low inter-individual variation between barn owl PTs (CV=

14.8% and 95% CI = 20.4-22.3 s) suggest that with the exception of one individual (101.6 s), barn owls were not significantly exposed to anticoagulant rodenticides. However, it does remain possible that sublethal or subclinical exposure may have occurred, which may be detected with these barn owl PTs are compared with a 'true' baseline value.

Although some of the discrepancy between the PT for control quail and for barn owls may be due to the sample handling conditions described above, it is most likely that the higher mean PT of barn owls is due to inter-species variation and the use of chicken thromboplastin. For biomonitoring purposes chicken thromboplastin is a reasonable reagent for avian species on the condition that a baseline PT is determined for the species in question, and ideally that the reagent is further tested against industry standard plasmas to ensure repeatability. It is unnecessary to determine the 'true' barn owl PT using homologous thromboplastin made from barn owl brain as this would not be conducted routinely. Further PT evaluations using industry standard thromboplastin reagents, manufactured for cat, dog or human PT determinations, is also warranted as they are stable and standardized, which would eliminate some inter-day variation in avian PT analyses. A biomonitoring program for anticoagulant rodenticide exposure of free-living birds requires careful selection of one or two species and a baseline PT measured in approximately 20 birds whose territories do not overlap the area of anticoagulant rodenticide use. Routine PT measurement in free-living owls, raptors, and birds delivered to rehabilitation centres, in combination with rodenticide liver residue analysis in

dead birds, would provide some indication of the level of anticoagulant rodenticide exposure and a measure of effect in a given region.

2.5 Tables

Table 2.1: Total number of mortalities which occurred after exposure to brodifacoum.

Dose (mg/kg)	Days post-exposure[a]			
	1	2-3	4-5	6-7
0	0	0	0	0
0.8	0	2	0	0
1.4	0	2	1	1
1.9	0	2	1	NA
2.5	0	0	5	NA

[a] Mortalities occurred either due to euthanasia for animal welfare purposes or directly as a result of exposure.

Table 2.2: Mean prothrombin times and inter-individual variation for the Japanese quail (*Coturnix japonica*) and Barn owl (*Tyto alba*) activated using chicken thromboplastin. Prothrombin times and inter-replicate variation of normal and abnormal mammalian coagulation controls (Pacific Hemostasis® Level 1 and 2, respectively) activated using chicken thromboplastin.

	Japanese quail	**Barn owl**	**Normal Control**	**Abnormal Control**
Mean PT (s) (95% CI)	13.2 (12.5-13.8)	21.4 (20.4-22.3)	107.1 (103.9-110.3)	182.3 (177.7-186.9)
PT Range: Min-Max (s)	10.8 - 15.4	12.6 – 30.5	96.0 - 117.0	161.6 - 201.3
Coefficient of variation	10.5 %	14.8 %	6.2 %	5.4 %

Table 2.3: Prothrombin times measured in avian species using homologous species, homologous family, or mammalian thromboplastin.

Species[a]	Prothrombin time (seconds)[bcd]	N	TF Source[e]	Reference
Avian plasma activated by homologous species thromboplastin (TF)				
Japanese quail (*Coturnix japonica*) – M	10.0 (0.29)	12	H	(Belleville et al., 1982)
Chicken (*Gallus gallus domesticus*)	9.4 (0.9)	6	H	(Morrisey et al., 2003)
Chicken (*Gallus gallus domesticus*) – LF	9.4 (0.1)	1200	H	(Doerr et al., 1975)
Chicken (*Gallus gallus domesticus*) – M	9.74 (0.14)	10	H	(Witlock and Wyatt, 1978)
Chicken (*Gallus gallus domesticus*) – M	9.8 (0.18)	32	H	(Fernandez et al., 1995)
Chicken (*Gallus gallus domesticus*)	11.0	2	H	(Griminger et al., 1970)
Chicken (*Gallus gallus domesticus*) – M	12.0	10	H	(Bigland and Triantaphyllopoulos, 1961)
Chicken (*Gallus gallus domesticus*) – M	12	8	H	(Stopforth, 1970)
Chicken (*Gallus gallus domesticus*)	13.02 (0.17)	60	H	(Timms, 1977)
Chicken (*Gallus gallus domesticus*) – LF	13.2 (0.3)	16	H	(Fernandez et al., 1995)
Chicken (*Gallus gallus domesticus*) – M	13.4	15	H	(Stopforth, 1970)
Chicken (*Gallus gallus domesticus*) – F	13.9	15	H	(Stopforth, 1970)
Chicken (*Gallus gallus domesticus*) – LF	25	6	H	(Bailey et al., 2005)
Chicken (*Gallus gallus domesticus*) – M	26.6 (0.3)	4	H	(Thomson et al., 2002)
Chicken (*Gallus gallus domesticus*) – M	27.1 (1.7)	4	H	(Thomson et al., 2002)
Chicken (*Gallus gallus domesticus*) – LF	31.5 (3.7)	4	H	(Thomson et al., 2002)
Chicken (*Gallus gallus domesticus*)	38.6 (3.3)	24	H	(Frost et al., 1999)
Ostrich (*Strutio camelus*)	73 (1.98)	48	H	(Frost et al., 1999)
Pigeon (*Columba livia*)	25 or L-11	10	H	(Tahira et al., 1977)
Kite (*Milvus migrans*)	18.2 or L-8.5	8	H	(Tahira et al., 1977)
Vulture (*Neophron percnopterus*)	16.2 or L-13.7	2	H	(Tahira et al., 1977)
Avian plasma activated by non-homologous avian species thromboplastin.				
Japanese quail (*Coturnix japonica*)	13.2 (0.30)	23	Chicken	This Study
California quail (*Callipepla californica*)	21.2	19	Chicken	(Blus et al., 1985)
Chicken (*Gallus gallus domesticus*)	90.1 (3.19)	23	Ostrich	(Frost et al., 1999)
Turkeys (*Meleagris gallopavo*)	12.40 (0.11)	50	Chicken	(Timms, 1977)
Ostrich (*Strutio camelus*)	54.9 (6.06)	24	Chicken	(Frost et al., 1999)
Amazon Parrot (*Amazona ventralis*)	10 (1.5)	6	Chicken	(Morrisey et al., 2003)
Cockatoo (*Cacatua alba*)	11.1 (0.8) – 13.6 (1.2)[d]	14	Chicken	(Morrisey et al., 2003)
Barn owl (*Tyto alba*)	21.4 (0.47)	46	Chicken	This Study
Golden eagle (*Aquila chrysaetos*)	23 (0.9)	7	Chicken	(Savarie et al., 1979)
Avian plasma activated by mammalian thromboplastin.				
Japanese quail (*Coturnix japonica*)	42.0 (1.15)	12	Human	(Belleville et al., 1982)
Chicken (*Gallus gallus domesticus*)	72	7	Rat	(Stopforth, 1970)
Chicken (*Gallus gallus domesticus*)	163	9	Human	(Stopforth, 1970)
Chicken (*Gallus gallus domesticus*)	145.3 (3.38)	21	Human	(Frost et al., 1999)
Ostrich (*Strutio camelus*)	190.5 (7.37)	41	Human	(Frost et al., 1999)
Pigeon (*Columba livia*)	169.7	11	Human	(Tahira et al., 1977)
Pigeon (*Columba livia*)	85.1	5	Rabbit	(Tahira et al., 1977)
Kite (*Milvus migrans*)	100.4	10	Human	(Tahira et al., 1977)
Vulture (*Neophron percnopterus*)	237.5	5	Human	(Tahira et al., 1977)
Black-headed Vulture (*Coragyps atratus*)	45.0-45.3	2	Human	(Weir-M. et al., 2004)

[a] M=male, F=female, LF=laying female.
[b] Mean (SEM) or *SEM calculated from SD/√n.
[c] L= Thromboplastin source was the lung.
[d] PT was evaluated on frozen sample.
[e] H = Homologous species thromboplastin.

Table 2.4: Time for which plasma or whole blood may be stored under different temperature conditions resulting in prothrombin time not significantly altered from fresh plasma.

Sample	21 to 23°C	4 to 6°C	-18 to -24°C	-70 to -80°C	Reference
Human Plasma	24 hours	24 hours	-	-	Heil et al., 1998
	24 hours*	6 hours; 24 hours[NS]	6 hours; 24 hours[NS]	-	Rao et al., 2000
	-	24 hours	1 month at -40°C	-	(Grau et al., 1999)
	24 hours	Do not use	2 weeks	6 months	NCCLS, 2003
	-	-	6 months (<5% variability); 1 year (<10% variability)	1 year (<5% variability); 2 years (<10% variability)	Woodhams et al., 2001
Whole human blood**	6 hours*	6 hours	-	-	Salvagno et al., 2008
	24 hrs	-	-	-	NCCLS, 2003
Avian Plasma	-	4 hours	None***	-	Timms, 1977
	None	-	3 months	-	Stopforth, 1970
	-	-	None	-	Doerr et al., 1975
	-	-	-	7 months[NS]	This study

[NS]Change in PT was not sufficient to change clinical interpretation of results.
None= The first timepoint tested resulted in a prothrombin time which varied from that of fresh plasma.
*Study did not report actual room temperature.
**Storage time prior to centrifugation
***Increase in PT was associated with free-thaw event, not the amount of time frozen.

2.6 Figures
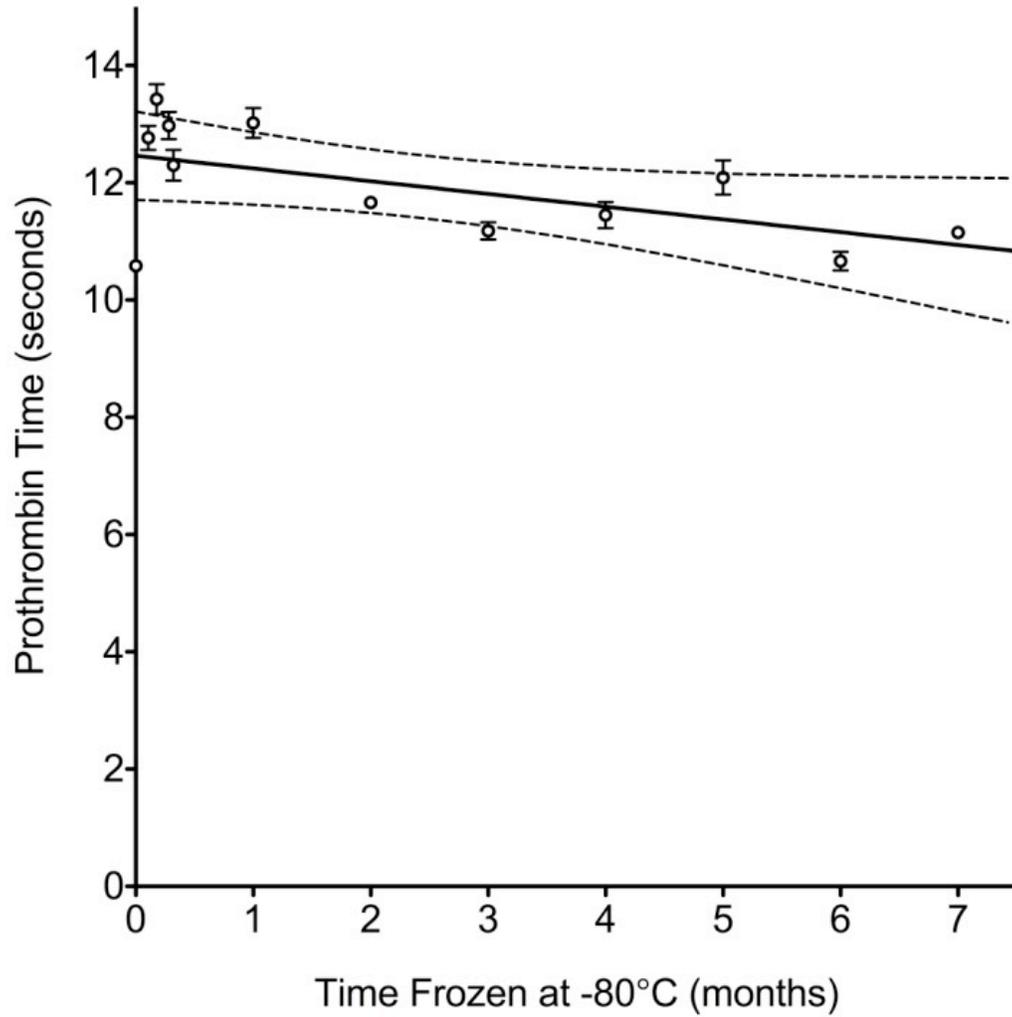
Figure 2.1: Prothrombin time measured in aliquots of plasma from one Japanese quail plasma pool after freezing for up to 7 months. Linear regression (slope= -0.0078 s/day; R^2=0.33) shows no significant trend (p=0.053) over time.
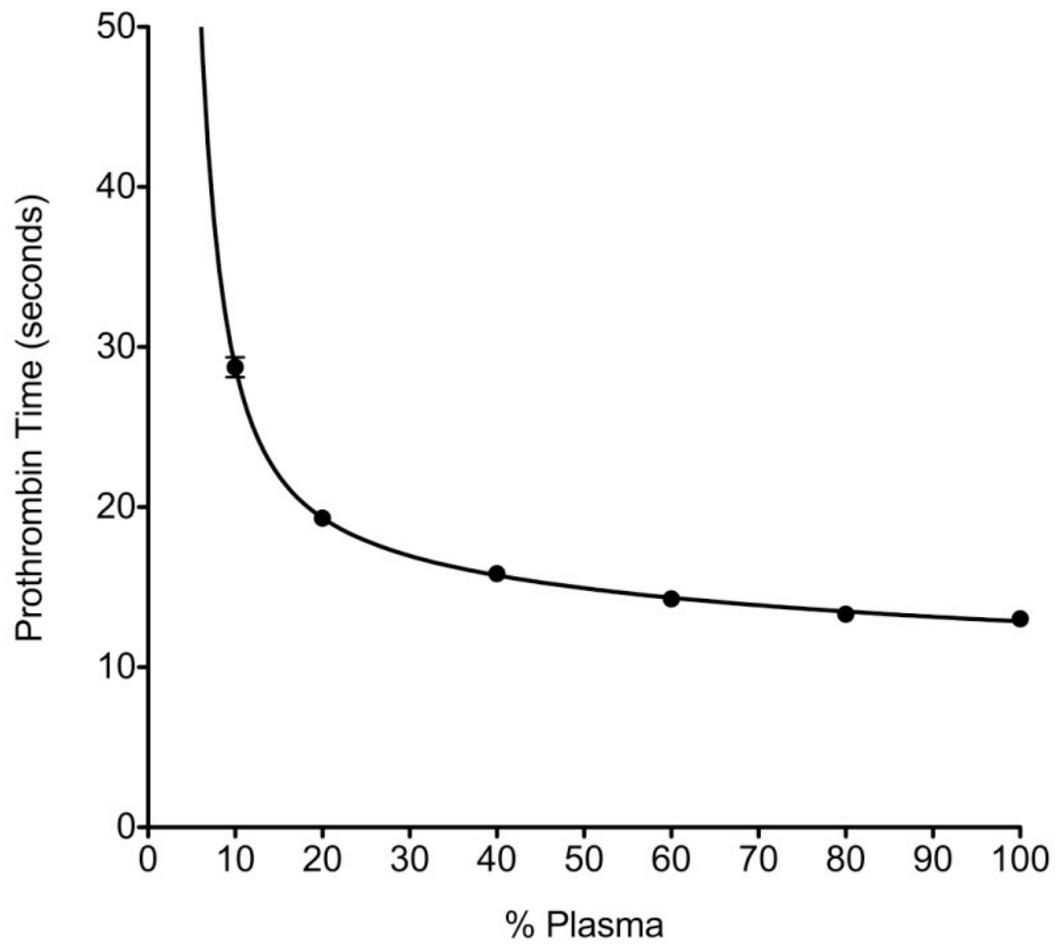
Figure 2.2: Prothrombin time measured in plasma dilutions using a nonlinear fit model ($y = 2148x^{-2.413} + 32.57x^{-0.2022}$; r^2=0.9996) to estimate the % normal clotting activity of a given sample based on prothrombin time.
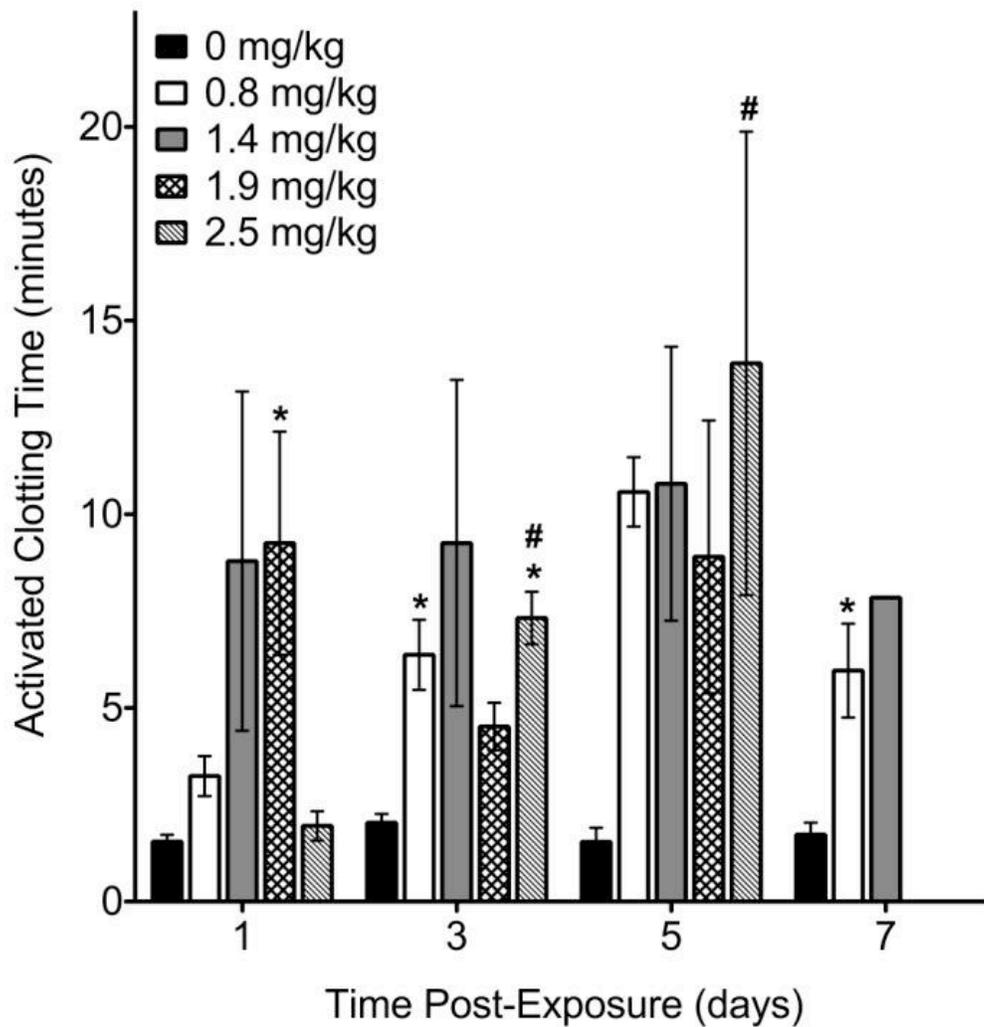
Figure 2.3: Activated clotting time (minutes) measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences ($p < 0.05$) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (∗), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#).
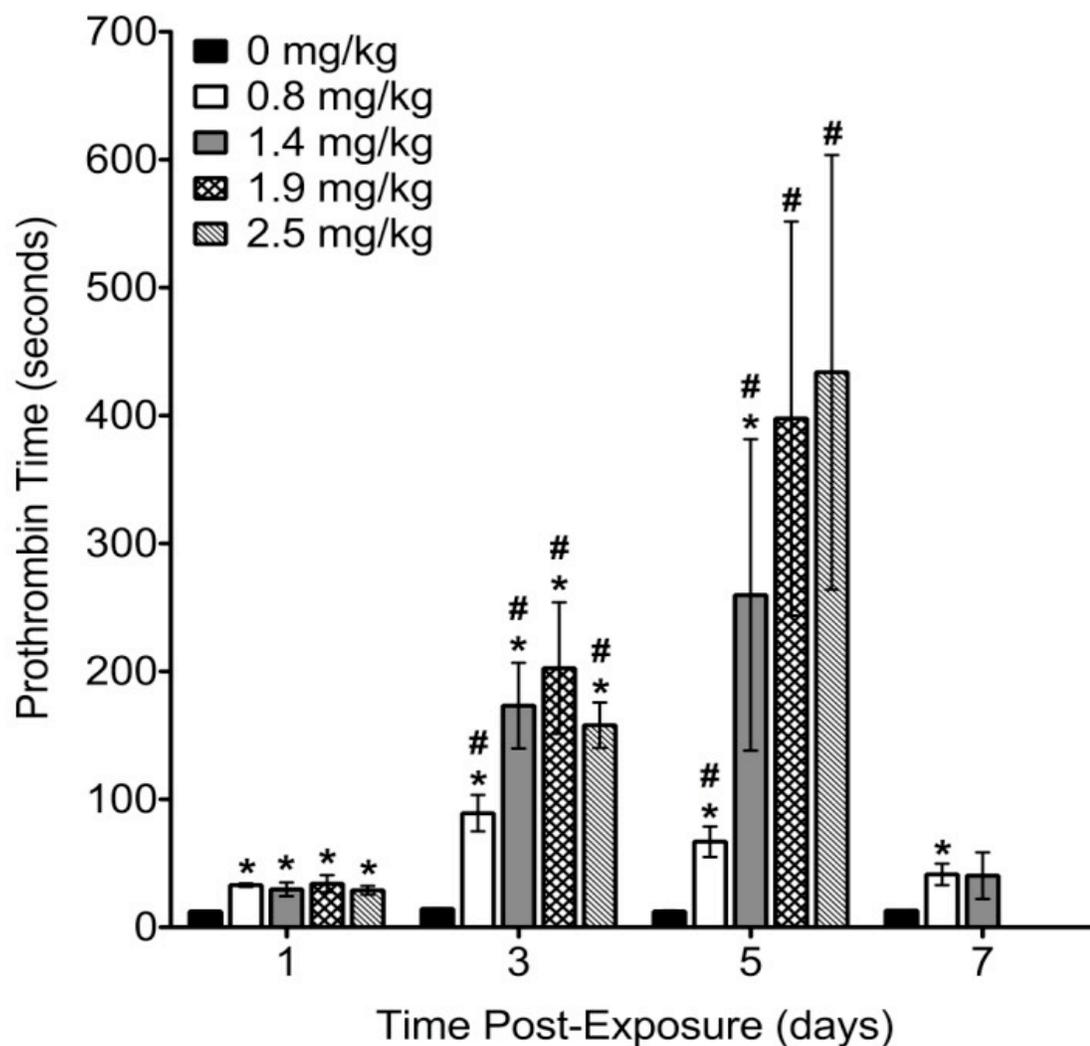
Figure 2.4: Prothrombin time measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences (p<0.05) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (∗), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#).
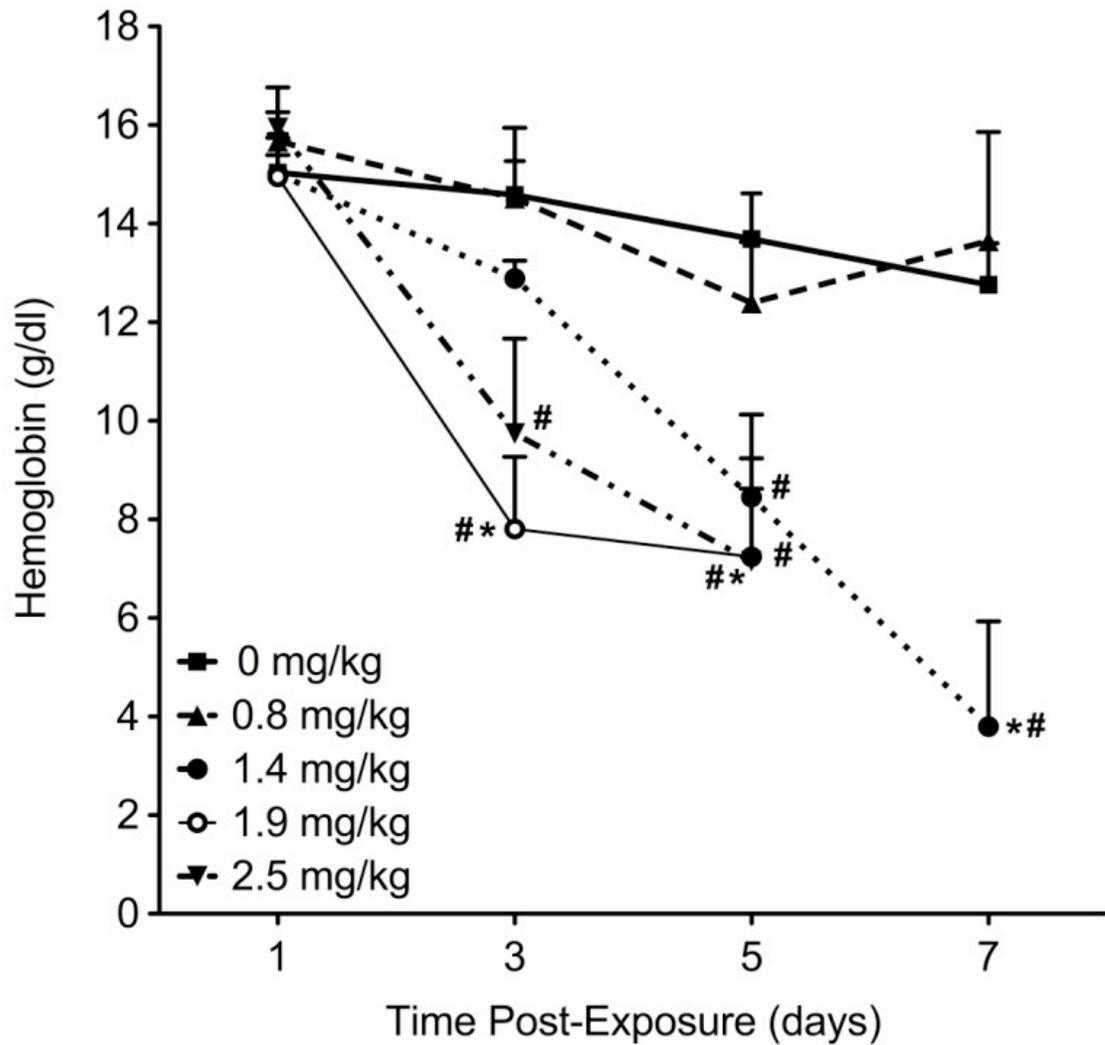
Figure 2.5: Hemoglobin (g/dl) measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences (p<0.05) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (✱), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#).
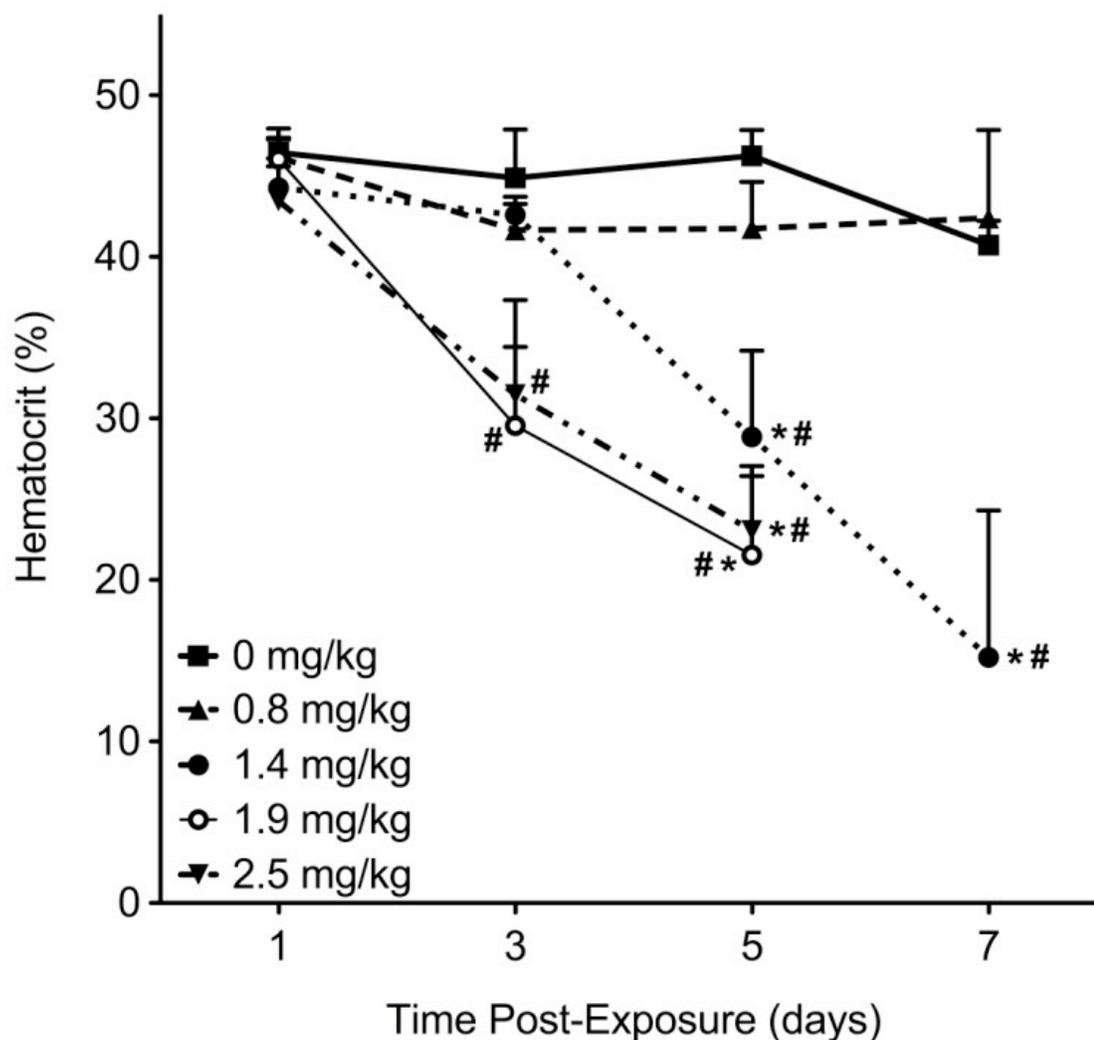
Figure 2.6: Hematocrit measured in Japanese quail (*Coturnix japonica*) after oral exposure to brodifacoum. Significant differences (p<0.05) occurred between dose groups in comparison to same-day control 1, 3, 5, and 7 days post-exposure (∗), and within each dose group after 3, 5, and 7 days post-exposure when compared with 1 day post-exposure (#).
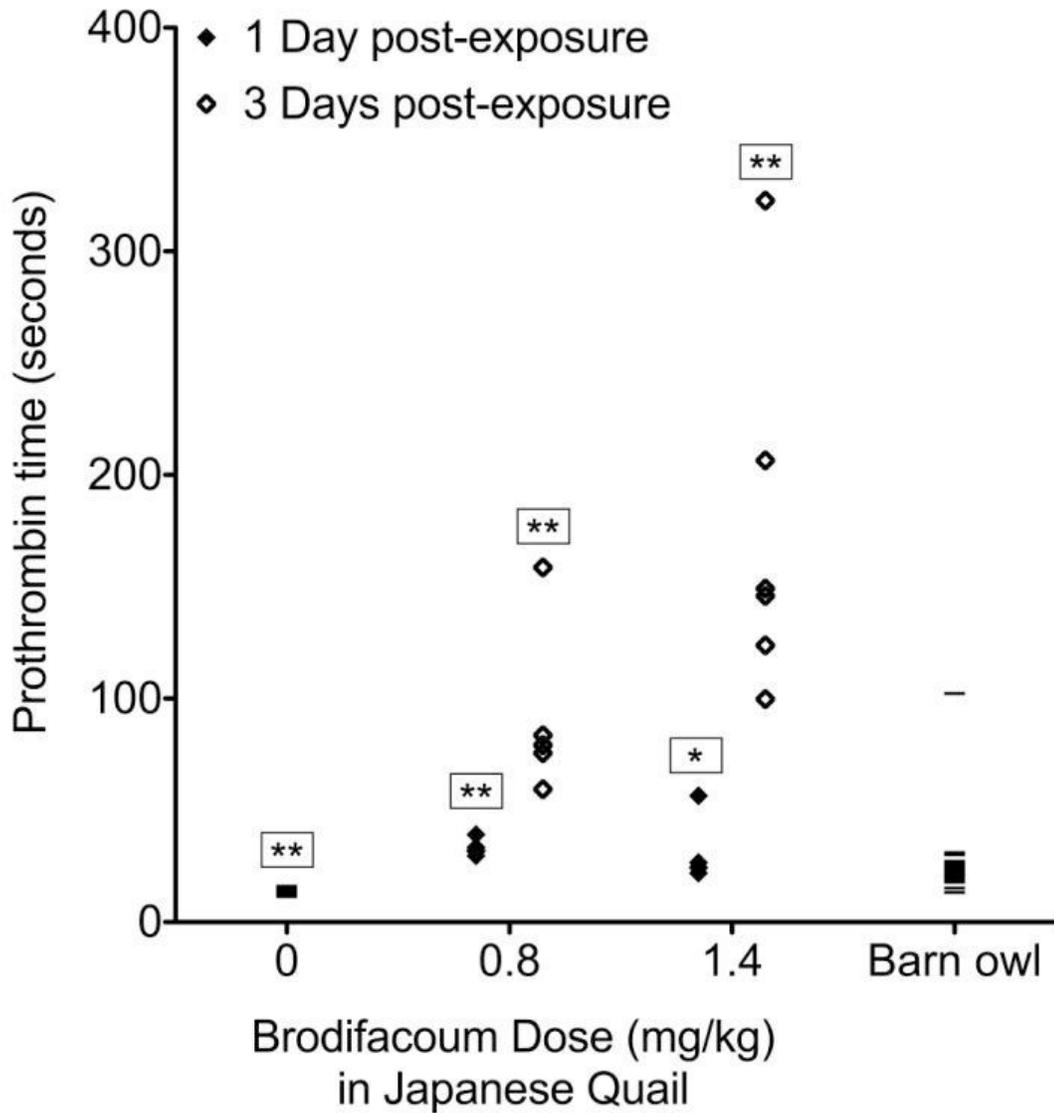
Figure 2.7: Prothrombin times measured in free-living Barn owls (n=47; *Tyto alba*) and in Japanese quail (*Coturnix japonica*) dosed with corn oil (n=21), or brodifacoum at 0.8 and 1.4 mg/kg, 1 and 3 days post-exposure (n=6 each). Significant differences are based on Bonferonni adjusted p-values of $p < 0.0005$ (★★) and $p < 0.05$ (★).

CHAPTER 3: CONCLUSIONS

Our current understanding of the mechanisms underlying avian coagulation is relatively limited as are the sub-lethal effects of anticoagulant rodenticides on avian species. Based on the results of the present study, we can a) make several recommendations as to how to improve application of assays to assess coagulation responses and b) make some suggestions for what additional, future research may be conducted to answer new questions which have surfaced in the present study. It is important to note, in particular, that non-coagulation effects of sub-lethal anticoagulant rodenticide exposure to wildlife remain largely unaddressed in the literature and a rationale for research recommendations is described below.

3.1 Contributions to our understanding of avian coagulation

The present study has made a number of significant contributions to the use of clotting time assays in the study of avian coagulation. The activated clotting time (ACT) assay was successfully validated in this study providing further evidence of the contact activation (intrinsic) pathway being present in avian species. This study has also provided a foundation for further study into the use of ACT as a viable screening test in avian species similar to its use in veterinary clinics to screen for cat and dog coagulation diseases. This simple test may have quite broad applications in avian species and be able to screen poultry for

aflatoxicosis, bleeding wild birds at wildlife rehabilitation centres and during island conservation projects following use of anticoagulant rodenticides.

The present study also provided a laboratory validation of the prothrombin time (PT) assay. We have shown that the PT assay can be an effective indicator of anticoagulant rodenticide exposure in a model avian species, the Japanese quail, responding in a dose- and time-dependent manner. It is essential to recognize that using chicken thromboplastin will likely result in each species having a different mean PT (Table 2.3) and establishing a baseline PT allows for direct comparison between that of healthy birds and those potentially exposed to anticoagulant rodenticides in the field. Clean venipuncture from a large blood vessel, such as the jugular vein, is also necessary to avoid causing any injury to endothelial cells within the vein and premature activation of the coagulation cascade. However, the option of using catheters for blood collection was not investigated. Based on the results from the quail study, immediate centrifugation (within 15 minutes) of whole blood and storage of plasma at -80°C for up to 7 months, is the recommended protocol to produce consistent PT results. These sampling and storage conditions should also be followed when collecting wild samples to minimize erroneous PT values and incorrect biomonitoring conclusions.

3.2 Studying anticoagulant rodenticide toxicity

In retrospect, some modifications to the study design could have improved the precision of the ACT results. Japanese quail are a convenient model species; however, their size limits the amount of blood which can be safely

obtained for coagulation testing. Based on commonly used ratios for the ACT test, 0.5 ml of whole blood is required per 3 mg diatomaceous earth (DE). Although that ratio may be used to cut down the blood volume, even a reduction by 50% would require 1.5 mg of DE to be weighed consistently into tubes, and microclot formation in this small volume may be difficult to see with the naked eye. Based on blood quantity limitations, it is recommended that in order to measure the dose-response for ACT with quail, no other blood assays be conducted in that study so intra-individual replicates can provide increased accuracy and precision. Sample sizes of 12 would also accommodate mortalities at lower doses, and focusing on only 2 dose groups in addition to the vehicle control group would still allow sampling to be done in a single day.   If a clear dose-response relationship can be determined for ACT with higher sample sizes and no false-negative results, this technique would be highly recommended as a field screening test of anticoagulant exposure due to the simplicity of the technique. Alternatively, the PT assay requires much less blood and is quite accurate, but does require testing to be done in the laboratory.

Based on observations throughout this study, the question arose of whether there are significant differences in how females respond to anticoagulant rodenticide exposure in comparison to males. Females may eliminate some of their brodifacoum into the egg, potentially causing them to be less susceptible to the amount consumed, compared to males. However, females must allocate resources to egg production and may incur injuries during mating, potentially increasing their susceptibility. It is unknown which, if any, of those factors have a

significant effect on the response of females to anticoagulant rodenticides in comparison to males. A study investigating those differences would ideally be designed similar to the one above with endpoints chosen as solely PT- or ACT-focused, large sample sizes, and only 2 doses.

The results of this study generated yet more questions as to the effects of brodifacoum at doses lower than 0.8 mg/kg. This dose provided interesting results with the PT being prolonged, but hematocrit and hemoglobin being unaffected. Although the vitamin K cycle was disrupted at the 0.8 mg/kg dose group, death would likely only occur due to injuries during the period of peak coagulation disruption resulting in significant blood loss. Also, at sublethal doses, hematocrit and hemoglobin do not appear to be good indicators of anticoagulant rodenticide exposure. A study using 0.8 mg/kg as the highest dose, as well as two lower doses (e.g. 0.05 and 0.4 mg/kg), may permit determination of a no observable adverse effects level (NOAEL) for PT as well as for reproduction and potentially offspring developmental endpoints. If this study does not measure ACT, only a small amount of blood would be required from each bird at sampling timepoints and non-lethal sampling might also provide information as to the time to PT recovery. However, at the 0.8 mg/kg dose there was some haemorrhaging and death, therefore repeat sampling at this dose is not recommended due to blood loss at the site of venipuncture. Finally, given the importance of blood collection technique, the question remains of whether catheters may be used for repeat blood collection in coagulation studies of avian species.

3.3 Future directions in anticoagulant rodenticide biomonitoring

The most recent proposed risk mitigation measures for anticoagulant rodenticides in Canada, released June 2009, prohibit brodifacoum and difethialone from being used in any outdoor application (PMRA, 2009). However, even if those requirements are implemented, there would still be a need for biomonitoring of other second-generation anticoagulant rodenticides (SGARs). SGARs such as bromadiolone may begin to pose an increased risk if usage becomes more widespread to replace outdoor use of brodifacoum and difethialone.

The comprehensive risk assessment conducted by the USEPA (2004) recommended not only that biomonitoring studies be conducted, but also that sublethal reproduction no observable adverse effects levels (NOAELs) be determined for each rodenticide. Warfarin is considered a teratogen based on mammalian research which shows growth plate deformations and other soft tissue calcifications (Vermeer and Schurgers, 2000). Warfarin and flocoumafen have also been shown to be transferred into the egg yolk and albumen from the laying hen (Eadsforth et al., 1993; Kammerer et al., 1998).   New bone malformation, and cartilage and arterial calcification caused by warfarin exposure, are believed to be due to undercarboxylation of osteocalcin and matrix gla protein (MGP) in those extrahepatic tissues (Haffa et al., 2000; Vermeer and Schurgers, 2000).   Osteocalcin, a negative regulator of bone formation, and MGP, a strong inhibitor of soft tissue calcification, have both been identified in the chicken (Hauschka et al., 1983; Wiedemann et al., 1998). The MGP has also

been shown to be a developmentally regulated inhibitor of cartilage mineralization in chicken hypertrophic chondrocyte cultures (Yagami et al., 1999). It is, therefore, possible that deposition of SGARs into the egg may disrupt bone formation of the developing chick at exposure levels lower than that required to disrupt coagulation of the laying female. In the present study, only the brodifacoum-exposed quail had a blood clot surrounding some of their primary and secondary vitellogenic follicles, suggesting that some egg-associated effects may occur. No avian studies have yet been conducted to investigate sublethal effects of brodifacoum, difethialone, or bromadiolone on reproduction. Currently, with the focus of rodenticide monitoring on residue analysis and necropsy findings, sublethal effects on reproduction would only be detected by conducting population surveys including reproductive success measurements. A laboratory study measuring a NOAEL for avian reproduction (as described above) would likely provide essential information as to the relative importance of reproductive effects in the protection of wildlife and may assist in directing the focus of future studies, biomonitoring and regulatory recommendations by wildlife toxicologists.

Additional questions arose regarding the spatial and temporal factors of the barn owl sampling. Although vole (*Microtus spp.*) availability is plentiful in the barn owl diet throughout the year, it has been shown that snow cover may reduce their ability to hunt this species (Campbell et al., 1987). When vole availability is reduced, alternate food sources, such as shrews (*Sorex spp.*), birds and larger prey items such as *Rattus spp.* become more important (Campbell et

al., 1987) potentially increasing exposure to rodenticides. During winter months, when vole populations may decrease until the breeding season in May (Campbell et al., 1987) and weather conditions are challenging, barn owls may hunt more near roost sites in barns and other buildings in rural agricultural areas when the proportion of mice and rats in the diet would increase. This seasonal fluctuation in diet may also correspond with increased use of rodenticides as commensal rodents move indoors, however no data has been obtained on the seasonal use of rodenticides. Sampling in this study was only conducted in rural agriculture areas where the diet is primarily composed of voles, however in urbanized habitats mice and rats have been shown to compose the largest proportion of the barn owl diet (Campbell et al., 1987), suggesting further sampling and PT measurements in these areas is warranted.

Biomonitoring of anticoagulant rodenticides is an arduous task due to the numerous confounding factors present when assessing exposure in free-living birds. Extrapolation from lab to field in addition to biomonitoring techniques are currently the strongest tools available for estimating risk to wildlife. This study suggests that only one of the barn owls (2%) tested during the nesting period was potentially exposed to brodifacoum (or another anticoagulant rodenticide) at a level sufficient to clinically disrupt coagulation. Finally, the present study was not designed to assess non-coagulation-related effects which may occur within barn owls, and this remains an important questions for future research.

REFERENCE LIST

Aguirre-Munoz A, Croll DA, Donlan CJ, Henry RW, Hermosillo MA, Howald GR, Keitt BS, Luna-Mendoza L, Rodriguez-Malagon M, Salas-Flores LM, Samaniego-Herrera A, Sanchez-Pacheco JA, Sheppard J, Tershy BR, Toro-Benito J, Wolf S, Wood B. 2008. High-impact Conservation: Invasive Mammal Eradications from the Islands of Western Mexico. *Ambio - Journal of Human Environment Research and Management* 37:101-107.

Albert CA, Wilson LK, Mineau P, Trudeau S, Elliott JE. 2009. Anticoagulant Rodenticides in Three Owl Species from Western Canada, 1988–2003. *Archives of Environmental Contaminants and Toxicology*, In press.

Andrusiak LA, Cheng KM. 1997. Breeding Biology of the Barn Owl (*Tyto alba*) in the Lower Mainland of British Columbia. NC-190. USDA Forest Service, Winnipeg, Manitoba.

ASTM. 1997. Standard Practice For Conducting Subacute Dietary Toxicity Tests With Avian Species. Report No. E857 - 05e1. American Society for Testing and Materials, West Conshohocken, PA.

Atkinson IAE. 2001. Introduced Mammals and Models For Restoration. *Biological Conservation* 99:81-96.

Atkinson IAE. 1985. The spread of commensal species of *Rattus* to oceanic islands and their effects on island avifaunas. In Moors PJ, ed, *Conservation of island birds: case studies for the management of threatened island species,* Technical Publication 3 ed, International Council for Bird Preservation, Cambridge, United Kingdom, pp 35-81.

Bachmann KA, Sullivan TJ. 1983. Dispositional and pharmacodynamic characteristics of brodifacoum in warfarin-sensitive rats. *Pharmacology* 27:281-288.

Bailey C, Fisher P, Eason CT. 2005. Assessing anticoagulant resistance in rats and coagulation effects in birds using small-volume blood samples. *Science for Conservation* 249:1-22.

Banfield DK, Irwin DM, Walz DA, MacGillivray RTA. 1994. Evolution of Prothrombin: Isolation and Characterization of the cDNAs Encoding Chicken and Hagfish Prothrombin. *Journal of Molecular Evolution* 38:177-187.

Barber DL, Colvin HW. 1980. Influence of dietary protein on the response of rats receiving toxic levels of warfarin. *Toxicology and Applied Pharmacology* 56:8-15.

Bardai G, Sunahara GI, Spear PA, Martel M, Gong P, Hawari J. 2005. Effects of Dietary Administration of CL-20 on Japanese Quail *Coturnix coturnix japonica*. *Archives of Environmental Contaminants and Toxicology* 49:215-222.

Bateman SW, Mathews KA. 1999. Comparisonn of Axillary and Heating Block methods of Activated Clotting Time (ACT) in Dogs. *The Journal of Veterinary Emergency and Critical Care* 9:79-82.

Batten, P, Bratt H. 1990. Brodifacoum: elimination from the tissues of rats following administration of single oral doses. Unpubl. report submitted to EPA by ICI Americas, Inc., Wilmington, DE. 65 pp. In US EPA, 2004.

Belleville J, Cornillon B, Paul J, Baguet J, Clendinen G, Eloy R. 1982. Haemostasis, Blood Coagualtion and Fibrinolysis in the Japanese Quail. *Comparative Biochemistry and Physiology Part A: Physiology* 71A:219-230.

Berkner KL. 2000. The Vitamin K-dependant Carboxylase. *Journal of Nutrition* 130:1877-1880.

Bigland CH. 1964. Blood Clotting Time of Five Avian Species. *Poultry Science* 43:1035-1039.

Bigland CH, Triantaphyllopoulos DC. 1961. Chicken Prothrombin, Thrombin, and Fibrinogen. *American Journal of Physiology* 200:1013-1017.

Blus LJ, Henny CJ, Grove RA. 1985. Effects of Pelletized Anticoagulant Rodenticides on California Quail. *Journal of Wildlife Diseases* 21:391-395.

Boyle CM. 1960. Case of Apparent Resistance of *Rattus norvegicus* to Anticoagulant Poisons. *Nature* 188:517.

Breckenridge AM, Cholerton S, Hart JAD, Park BK, Scott AK. 1985. A study of the relationship between the pharmacokinetics and the pharmacodynamics of the 4-hydroxycoumarin anticoagulants warfarin, difenacoum and brodifacoum in the rabbit. *British Journal of Pharmacology* 84:81-91.

Breckenridge A, L'E Orme M. 1972. The plasma half lives and the pharmacological effect of the enantiomers of warfarin in rats. *Life Sciences* 11:337-345.

Bunn DS, Warburton AB, Wilson RDS. 1982. *The Barn owl.* Buteo Books, Vermillion, SD.

Campbell EC, Campbell RW. 1984. Status of the Barn Owl (*Tyto alba*) in Canada. Committee on the Status of Endangered Wildlife in Canada (COSEWIC), Canadian National Federation, Ottawa, ON.

Campbell RW, Dawe NK, McTaggart-Cowan I, Cooper JM, Kaiser GW, McNall MCE. 1990. *The Birds of British Columbia, Vol. 2, Nonpasserines: Diurnal Birds of Prey through Woodpeckers.* Royal British Columbia Museum, Victoria, British Columbia.

Campbell RW, Manuwal DA, Harestad AS. 1987. Food habits of the common Barn Owl in British Columbia. *Canadian Journal of Zoology* 65:578-586.

Campbell TW, Ellis CK. 2007. Hematology of Birds. In Campbell TW, Ellis CK, eds, *Avian and Exotic Hematology and Cytology,* 3rd ed., Blackwell Publishing, Ames, Iowa, USA, pp 3-50.

Carpenter NA. 2000. Anseriform and galliform therapeutics. *Veterinary Clinics of North America: Exotic Animal Practice* 3:1-17.

CDC. 2006. Diseases From Rodents. Department of Health and Human Services, Center for Disease Control and Prevention, Atlanta, GA, USA. http://www.cdc.gov/rodents/diseases/ (August 10, 2009)

Cheng KM, Bennett DC, Mills AD. in press. Chapter 45: The Japanese quail. In *UFAW Handbook On The Care And Management Of Laboratory And Other Research Animals,* 8th ed, Universities Federation for Animal Welfare, .

Coenen TMM, Enninga IC, Cave DA, van der Hoeven JCM. 1994. Hematology and serum biochemistry of Japanese quail fed dietary tri-*n*-butyltin oxide during reproduction. *Archives of Environmental Contamination and Toxicology* 26:227-233.

Colvin HW, Wang WL. 1974. Toxic Effects of Warfarin in Rats Fed Different Diets. *Toxicology and Applied Pharmacology* 28:337-348.

COSEWIC. 2009. COSEWIC Status Reports in Preparation. Updated September 18, 2009. http://www.cosewic.gc.ca.proxy.lib.sfu.ca/eng/sct2/sct2_4_e.cfm

COSEWIC. 2001. Barn Owl. Updated November, 2001. http://www.cosewic.gc.ca.proxy.lib.sfu.ca/eng/sct1/searchform_e.cfm.

Cox P, Smith RH. 1992. Rodenticide ecotoxicology: Pre-lethal effects of anticoagulants on rat behaviour. *Proceedings of the Fifteenth Vertebrate Pest Conference*.

Cox PR, Smith RH. 1990. Rodenticide Ecotoxicology: Assessing Non-Target Population Effects. *Functional Ecology* 42:315-320.

Cronin DE. 1979. Warfarin resistance in house mice (*Mus musculus* L.) in Vancouver, British Columbia. MPM Thesis. Simon Fraser University, Burnaby, BC, Canada.

Davidson CJ, Hirt RP, Lal K, Snell P, Elgar G, Tuddenham EGD, McVey JH. 2003. Molecular Evolution of the Vertebrate Blood Coagulation Network. *Thrombosis and Haemostasis* 89:420-428.

Doerr JA, Hamilton PB. 1981. New Evidence for Blood Coagulation in Chickens. *Poultry Science* 60:237-242.

Doerr JA, Wyatt RD, Hamilton PB. 1976. Impairment of Coagulation Function During Aflatoxicosis in Young Chickens. *Toxicology and Applied Pharmacology* 35:437-446.

Doerr JA, Wyatt RD, Hamilton PB. 1975. Investigation and standardization of prothrombin times in chickens. *Poultry Science* 54:969-980.

Drabkin DL, Austin JH. 1935. Spectrophotometric studies: V. A technique for the analysis of undiluted blood and concentrated hemoglobin solutions. *The Journal of Biological Chemistry* 112:105-115.

Duxbury BM, Poller L. 2001. State-of-the-Art Review: The Oral Anticoagulant Saga: Past, Present, and Future. *Clinical and Applied Thrombosis/Hemostasis* 7:269-275.

Eadsforth CV, Gray A, Huckle KR, Inglesfield C. 1993. The dietary toxicity of flocoumafen to hens: Elimination and accumulation following repeated oral administration. *Pesticide Science* 38:17-25.

Eason CT, Murphy EC, Wright GRG, Spurr EB. 2002. Assessment of Risks of Brodifacoum to Non-target Birds and Mammals in New Zealand. *Ecotoxicology* 11:35-48.

Elliott AC. 1995. Rodenticides. In Godfrey CRA, ed, *Agrochemicals From Natural Products,* Marcel Dekker inc., New York, USA, pp 341-368.

Elliott JE, Kennedy SW, Lorenzen A. 1997. Comparative Toxicity of Polychlorinated Biphenyls to Japanese Quail (*Coturnix c. japonica*) and American Kestrels (*Falco sparverius*). *Journal of Toxicology and Environmental Health, Part A: Current Issues* 51:57-75.

Environment Canada, BC Ministry of Environment, prepared by ENKON Environmental Limited. 2005. Survey of Pesticide Use in British Columbia: 2003. Project No.1004-011.

Fernandez A, Verde MT, Gomez J, Gascon M, Ramos JJ. 1995. Changes in the prothrombin time, haematology and serum proteins during experimental aflatoxicosis in hens and broiler chickens. *Research in Veterinary Science* 58:119-122.

Fisher P, O'Connor C, Wright G, Eason CT. 2003. Persistence of four anticoagulant rodenticides in the livers of laboratory rats. Report No. 139. New Zealand Department of Conservation, Wellington.

Franz SC, Padula CM. 1980. Recent Developments in anticoagulant Rodenticide Resistance Studies: Surveillance and Application in the United States. *Proceedings of the 9th Vertebrate Pest Conference*.

Fraser DF, Harper WL, Cannings SG, Cooper JM. 1999. Barn owl (*Tyto alba*). In *Rare Birds of British Columbia,* Wildlife Branch and Resources Inventory Branch, Victoria, B.C., pp 107-108.

Frost CL, Naudé RJ, Oelofsen W, Jacobson B. 1999. Comparative blood coagulation studies in the ostrich. *Immunopharmacology* 45:75-81.

Gebauer M. 2007. Synthesis and structure–activity relationships of novel warfarin derivatives. *Bioorganic and Medicinal Chemistry,* 15:2414-2420.

Godfrey MER. 1986. An Evaluation of the Acute Oral Toxicity of Brodifacoum to Birds. *Proceedings of the Twelfth Vertebrate Pest Conference.*

Godfrey MER. 1985. Non-target and secondary poisoning hazards of "second generation" anticoagulants. *Acta Zoologica Fennica* 173:209-212.

Godfrey WE. 1986. *The Birds of Canada.* National Museum of Canada, Ottawa, Ontario, Canada.

Grau E, Tenias JM, Olaso MA, Ferrando I, Juan MT, Pastor E, Perez A, Real E. 1999. Monitoring oral anticoagulant treatment from plasma stored for up to 48 hours and frozen plasma. *Haematologica* 84:633-636.

Gray A, Eadsforth CV, Dutton AJ, Vaughan JA. 1994a. The toxicity of three second-generation rodenticides to Barn Owls. *Pesticide Science* 42:179 - 184.

Gray A, Eadsforth CV, Dutton AJ, Vaughan JA. 1994b. Non-invasive method for monitoring the exposure of Barn owls to second-generation rodenticides. *Pesticide Science* 41(4): 339 – 343.

Gray A, Eadsforth CV, Dutton AJ, Vaughan JA. 1992. Toxicity of second generation rodenticides to Barn Owls. *British Crop Protection Council, Farnham (UK)*: 781-786.

Greaves JH, Ayres PB. 1967. Heritable Resistance to Warfarin in Rats. *Nature* 2165:877-878.

Griminger P. 1986. Coagulation. In Sturkie PD, ed, *Avian Physiology,* Fourth ed, Springer-Veralg, New York, NY, pp 121-125.

Griminger P, Shum YS, Budowski P. 1970. Effect of Dietary Vitamin K on Avian Brain Thromboplastin Activity. *Poultry Science* 49:1681-1686.

Hadler MR, Shadbolt RS. 1975. Novel 4-hydroxycoumarin anticoagulants active against resistant rats. *Nature* 253:275-277.

Haffa A, Krueger D, Bruner L, Engelke J, Gundberg C, Akhter M, Binkley N. 2000. Diet- or Warfarin-Induced Vitamin K Insufficiency Elevates Circulating Undercarboxylated Osteocalcin Without Altering Skeletal Status in Growing Female Rats. *Journal of Bone and Mineral Research* 15:872-878.

Harr KE, Myers G. In press. Overview of Avian Hemostasis. In Weiss D, Wardrop J, eds, *Schalm's Veterinary Hematology,* 6th ed, John Wiley & Sons Inc.

Hauschka PV, Frenkel J, DeMuth R, Gundberg CM. 1983. Presence of Osteocalcin and Related Higher Molecular Weight 4-Carboxyglutamic Acid-containing Proteins in Developing Bone. *The Journal of Biological Chemistry* 258:176-182.

Hegdal PL, Colvin BA. 1988. Potential Hazard to Eastern Screech-Owls and Other Raptors of Brodifacoum Bait Used for Vole Control. *Environmental Toxicology and Chemistry* 7:245-260.

Heil W, Grunewald R, Amend M, Heins M. 1998. Influence of Time and Temperature on Coagulation Analytes in Stored Plasma. *Clinical Chemistry and Laboratory Medicine* 36:459-462.

Hendley P, McIntosh S. 1982. A Crop Rotation Study. RJ0233. Imperial Chemical Industries, Amsterdam, the Netherlands.

Hindmarch S. In prep. How does land use influence the distribution and breeding success of barn owl (*Tyto alba*) in the lower mainland, British Columbia. *M. Sc.* Thesis. Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada.

Howald GR, Donlan CJ, Galvan JP, Russell JC, Parkes J, Samaniego A, Wang Y, Veitch D, Genovesi P, Pascal M, Saunders A, Tershy B. 2007. Invasive Rodent Eradication on Islands. *Conservation Biology* 21:1258-1268.

Howald GR, Mineau P, Elliott JE, Cheng KM. 1999. Brodifacoum Poisoning of Avian Scavengers During Rat Control on a Seabird colony. *Ecotoxicology* 8:431-447.

Howald GR. 1997. The Risk of Non-Target Species Poisoning from Brodifacoum Used to Eradicate Rats From Langara Island, British Columbia, Canada. Ph.D. Thesis. Department of Animal Science, University of British Columbia, Vancouver, British Columbia.

Howard WE, Wagnon KA, Bently JR. 1959. Competition between ground squirrels and cattle for range forage. *Journal of Range Management* 12:110-115.

Ikeda M, Conney AH, Burns JJ. 1968. Stimulatory Effect of Phenobarbital and Insecticides on Warfarin Metabolism in the Rat. *The Journal of Pharmacology and Experimental Therapeutics* 162:338-343.

Jackson WB, Kaukeinen DE. 1972. Resistance of Wild Norway Rats in North Carolina to Warfarin Rodenticide. *Science* 176(4041):1343-1344.

James SB, Raphael BL, Cook RA. 1998. Brodifacoum Toxicity and Treatment in a White-Winged Wood Duck (*Cairina scutulata*). *Journal of Zoo and Wildlife Medicine* 29:324-327.

Jansing RL, Chao ES, Kaminsky LS. 1992. Phase II Metabolism of Warfarin in Primary Culture of Adult Rat Hepatocytes. *Molecular Pharmacology* 41:209-215.

Jones HP, Tershy BR, Zavaleta ES, Croll DA, Keitt BS, Finkelstein ME, Howald GR. 2008. Severity of the Effects of Invasive Rats on Seabirds: A Global Review. *Conservation Biology* 22:16-26.

Jones HP, William HR, Howald GR, Tershy BR, Croll DA. 2006. Predation of artificial Xantus's murrelet (*Synthliboramphus hypoleucus scrippsi*) nests before and after black rat (*Rattus rattus*) eradication. *Environmental Conservation* 32:320-325.

Kaminsky LS, Zhang Z.-Y. 1997. Human P450 metabolism of warfarin. *Pharmacology and Therapeutics* 73:67-74.

Kammerer M, Pouliquen H, Pinault L, Loyau M. 1998. Residues depletion in egg after warfarin ingestion by laying hens. *Veterinary and Human Toxicology* 40:273-275.

Kaukeinen DE, Spragins CW, Hobson JF. 2000. Risk-Benefit Considerations In Evaluating Commensal Anticoagulant Rodenticide Impacts to Wildlife. *Ninteenth Vertebrate Pest Conference,* Mission Valley Hilton Hotel, San Diego, California, March 6-9, 2000, pp 245-256.

Kaukeinen DE, Rampaud M. 1986. A review of brodifacoum efficacy in the U.S. and worldwide. *Proceedings of the Twelfth Vertebrate Pest Conference*.

Keran D. 1981. The incidence of man-caused and natural mortalities to raptors. *Raptor Research* 15:108-112.

Kijlstra A, Meerburg BG, Cornelissan J, De Craeye S, Vereijken P, Jongert E. 2008. The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. *Veterinary Parasitology* 156:183-190.

Knudsen EI. 1981. The hearing of the Barn owl. *Scientific American* 245:112-125.

Kress SW. 1998. Applying research for effective management: case studies in seabird restoration. In Marduff JM, Sallabanks R, eds, *Avian Conservation: Research and Management,* Island Press, Washington, DC, US, pp 141-154.

Kurachi K, Kurachi S, Hamada T, Suenaga E, Bolotova T, Solovieva E. 2008. Part 7: Age-Related Homeostasis and Hemostatic System. In Tanaka K, Davie EW, eds, Springer, Japan, pp 427-438.

Laliberte R, Chakrabarti S, Brodeur J. 1976. The Influence of Fasting and Stress on the Response of Rats to Warfarin. *Journal of Pharmacology and Experimental Therapeutics* 196:194-203.

Lamboo M, Poland DCW, Eikenboom JCJ, Harvey MS, Groot E, Brand A, de Vries RRP. 2007. Coagulation parameters of thawed fresh-frozen plasma during storage at different temperatures. *Transfusion Medicine* 17:182-186.

Larson AE, Friedman PA, Suttie JW. 1981. Vitamin K-Dependent Carboxylase, Stoichiometry Of Carboxylation And Vitamin K 2,3-Epoxide Formation. *The Journal of Biological Chemistry* 256:11032-11035.

Lenton GM. 1984. The feeding and breeding ecology of Barn owls *Tyto alba* in peninsula Malaysia. *International Journal of Avian Science* 126:551-575.

Link KP, Berg D, Barker WM. 1965. Partial Fate of Warfarin in the Rat. *Science* 150:378.

Littin KE, Mellor DJ. 2005. Strategic animal welfare issues: ethical and animal welfare issues arising from the killing of wildlife for disease control and environmental reasons. *Revue Scientifique et Technique-Office International des Epizooties* 24(2):767-782.

Lund M. 1984. Resistance to the second-generation anticoagulant rodenticides. *Proceedings of the Eleventh Vertebrate Pest Conference*.

Lund M. 1964. Resistance to warfarin in the common rat. *Nature* 203:778.

MacDonald DW. 2001. The new encyclopedia of mammals, 2nd Ed. 961 pp.

Mackman N, Tilley RE, Key NS. 2007. Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 27:1687-1693.

Mann K, Nesheim M, Church W, Haley P, Krishnaswamy S. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* 76:1-16.

Marti CD. 1992. Barn Owl (*Tyto alba*). In Poole A, Stettenheim P, Gill F, eds, *The Birds of North America,* Vol 1. Academy of Natural Science and American Ornithologists' Union, Philadelphia, PA and Washington, DC, pp 1-15.

Massey JG, Valutis L, Marzluff J, Powers LV. 1997. The Anticoagulant Diphacinone's Effect on Crow Prothrombin Time. *Proceedings of the Annual Conference of the Association of Avian Veterinarians,* Bedford, Texas, pp 97-98.

Meerburg BG, Frans WAB, Kijlstra A. 2008. Perspective: The ethics of rodent control. *Pest Management Science* 64:1205-1211.

Mendenhall VM, Pank LF. 1980. Secondary poisoning of owls by anticoagulant rodenticides. *Wildlife Society Bulletin* 8:311-315.

Michal G. 1999. Blood Coagulation and Fibrinolysis. In *Biochemical Pathways, An Atlas of Biochemistry and Molecular Biology,* John Wiley and Sons, Inc., New York, NY., pp 251-160.

Middleton DJ, Watson ADJ. 1978. Activated Coagulation Times of Whole Blood in Normal Dogs and Dogs with Coagulopathies. *Journal of Small Animal Practice* 19:417-422.

Morrisey JK, Paul-Murphy J, Fialkowski JP, Hart A, Darien BJ. 2003. Estimation of Prothrombin Times of Hispaniolan Amazon Parrots (*Amazona ventralis*) and Umbrella Cockatoos (*Cacatua alba*). *Journal of Avian Medicine and Surgery* 17:72-77.

Mosterd JJ, Thijssen HHW. 1991. The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats.. *British Journal of Pharmacology* 104:531-535.

Murray M, Tseng F. 2008. Diagnosis and Treatment of Secondary Anticoagulant Rodenticide Toxicosis in a Red-tailed Hawk (*Buteo janaicensis*). *Journal of Avian Medicine and Surgery* 22:41-46.

Newton I, Wyllie I, Freestone P. 1990. Rodenticides in British Barn Owls. *Environmental Pollution* 68:101-117.

Parmar G, Batten PL. 1987. Detection of an anticoagulant binding site in rat liver microsomes and its relevance to the anticoagulant effect. *Human Toxicology* 6:432.

Parmar G, Bratt H, Moore R, Batten PL. 1987. Evidence for a common binding site *in vivo* for the retention of anticoagulants in rat liver. *Human Toxicology* 6:431.

Pelfrene AF. 2001. Rodenticides. In Krieger RI, ed, *Handbook of Pesticide Toxicology,* 2nd ed, Vol 2. pp 1793-1836.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics* 52:273-288.

PMRA. 2009. Proposed Risk Mitigation Measures for Eight Rodenticides. REV2009-05. Pest Management Regulatory Agency, Health Canada, Ottawa, Ontario, Canada.

PMRA. 2006. Re-evaluation Decision Document. Brodifacoum, Bromadiolone, Chlorophacinone, Diphacinone and Warfarin. RRD2006-11. Pest Management Regulatory Agency, Health Canada, Ottawa, Ontario, Canada.

PMRA. 2004. Re-evaluation of Brodifacoum, Bromadiolone, Chlorophacinone, Diphacinone and Warfarin. PACR2004-27. Pest Management Regulatory Agency, Health Canada, Ottawa, Ontario, Canada.

Poche RM, Mach JJ. 2001. Wildlife Primary and Secondary Toxicity Studies with Warfarin. In Johnston JJ, ed, *Pesticides and Wildlife,* Symposium ed, Vol 771. American Chemical Society, Washington, DC, pp 181-196.

Rao LV, Okorodudu AO, Petersen JR, Elghetany MT. 2000. Stability of prothrombin time and activated partial thromboplastin time tests under different storage conditions. *Clinica Chimica Acta* 300:13-21.

Rost S, Fregin A, Hünerberg M, Bevans CG, Müller CR, Oldenburg J. 2005. Site-directed mutagenesis of coumarin-type anticoagulant-sensitive VKORC1. *Thrombosis and Haemostasis* 94:780-786.

Salvagno GL, Lippi G, Montagnana M, Franchini M, Poli G, Guidi GC. 2008. Influence of temperature and time before centrifugation of specimens for routine coagulation testing. *International Journal of Laboratory Hematology* 31(4):462-467.

Saunders A, Blaffart H, Morley C, Kuruyawa J, Masibalavu V, Seniloli E. 2007. A "Community" Approach To Invasive Species Management: Some Pacific Case Studies. *Managing Vertebrate Invasive Species: Proceedings of an International Symposium,* Fort Collins, Colorado, August 7-9, 2007, pp 29-33.

Savarie PJ, Hayes DJ, Bride RT, Roberts JD. 1979. Efficacy and Safety of Diphacinone as a Predacide. In Kenaga EE, ed, *Avian and mammalian wildlife toxicology,* Special Technical Publication 693 ed, American Society for Testing and Materials, pp 67-79.

Schmaljohn C, Hjelle B. 1997. Hantaviruses: A Global Disease Problem. *Emerging Infectious Diseases* 3(2): 95-104.

Sheffield SR. 1997. Owls as Biomonitors of Environmental Contamination. *United States Department of Agriculture Forest Service General Technical Report,* pp.383-398.

Shirer M. 1992. In Poison's Defence. *Terra Nova* 17:3.

Shlosberg A, Booth L. 2006. Veterinary and Clinical Treatment of Vertebrate Pesticide Poisoning – a Technical Review. Landcare Research, Lincoln, New Zealand.

Silverman, R.B. 1980. A Model For A Molecular Mechanism Of Anticoagulant Activity Of 3-Substituted 4-Hydroxycoumarins. *Journal of the American Chemistry Society*, 102(16): 5421-5423.

Singleton GR, Leirs H, Hinds LA, Zhang Z. 1999. Ecologically-based management of rodent pests – re-evaluating our approach to an old problem. In Singleton GR, Hinds LA, Leirs H, Zhang Z, eds, *Ecologically-based Management of Rodent Pests,* Australian Centre for International Agricultural Research, Canberra, Australia, pp 17–29.

Smith C, Kerr K, Sadripour A. 2008. Pesticide Exports from U.S. Ports, 2001–2003. *International Journal of Occupational and Enviornmental Health* 14:176-186.

Stevens JEB, Hill IR. 1979. Brodifacoum Leaching on Thick-Layer Chromatograms. Imperial Chemical Industries Plant Protection Division Report No. RJ0072B, October 1979 (Unpublished). In: Kaiser et al., 1997.

Stone WB, Okoniewski JC, Stedelin JR. 2003. Anticoagulant Rodenticides and Raptors: Recent Findings from New York, 1998–2001. *Bulletin of Environmental Contamination and Toxicology* 70:34-40.

Stopforth A. 1970. A Study of Coagulation Mechanism in Domestic Chickens. *Journal of Comparative Pathology* 80:525-533.

Tahira N, Dube B, Agrawal GP. 1977. Blood Coagulation Studies in some Wild Indian Birds: Effect of Different Tissue Thromboplastins. *Journal of Comparative Pathology* 87:451-457.

Taylor RH, Thomas BW. 1989. Eradication of Norway Rats (*Rattus norvegicus*) from Hawea Island, Fiordland, using Brodifacoum. *New Zealand Journal of Ecology* 12:23-32.

Thomson JM. 1984. Chapter 33: Specimen Collection for Blood Coagulation Testing. In Koepke JA, ed, *Laboratory Hematology,* Vol 2. Churchill Livingstone, NY, USA.

Thomson AE, Squires EJ, Gentry PA. 2002. Assessment of factor V, VII and X activities, the key coagulant proteins of the tissue factor pathway in poultry plasma. *British Poultry Science* 43:313.

Timms L. 1977. The Estimation of Prothrombin Time of Chicken and Turkey Plasma Using a Phenol-Saline Thromboplastin or Russell Viper Venom. *British Veterinary Journal* 133:623-628.

Towns DR, Broome KG. 2003. From small Maria to massive Campbell: forty years of rat eradications from New Zealand islands. *New Zealand Journal of Zoology* 30:377-398.

US EPA. 2008. Risk Mitigation Decision for Ten Rodenticides. Office of Pesticides Programs, Environmental Fate and Effects Division, Washington, DC, USA.

US EPA. 2004. Potential Risks of Nine Rodenticides to Birds and Nontarget Mammals: A Comparative Approach. Office of Pesticides Programs, Environmental Fate and Effects Division, Washington, DC, USA.

Vermeer C, Schurgers LJ. 2000. A comprehensive review of vitamin K and vitamin K antagonists. *Blood Stasis and Thrombosis* 14:339-353.

Wadelius, M. and Pirmohamed, M. 2007. Pharmacogenetics of warfarin: Current status and future challenges. *The Pharmacogenomics Journal* 7:99-111.

Walker LA, Turk A, Long SM, Wienburg CL, Best J, Shore RF. 2008. Second generation anticoagulant rodenticides in tawny owls (*Strix aluco*) from Great Britain. *Science of the Total Environment* 392:93-98.

Wallin R, Sane DC, Hutson SM. 2003. Vitamin K 2,3-epoxide reductase and the vitamin K-dependent gamma-carboxylation system. *Thrombosis Research* 108:221 – 226.

Warburton PA, O'Connor CE. 2004. Research on Vertebrate Pesticides and Traps: Do Wild Animals Benefit? *Fourth World Congress on Alternatives to Animal Use in Science. ATLA* 32:229–234.

Weir-M. J, Acurero Z, Salas-A. R, Arteaga-Vizcaino M. 2004. Blood coagulation factors in the black headed vulture (*Coragyps atratus*), a potential animal model for the study of haemostasis. *Thrombosis Research* 113:269-273.

Whitlon DS, Sadowski JA, Suttie JW. 1978. Mechanism of coumarin action: Significance of vitamin K epoxide reductase inhibition. *Biochemistry* 17:1371-1377.

WHO. 1995. Brodifacoum Health and Safety Guide. 93. World Health Organization, Geneva.

Wiedemann M, Trueb B, Belluoccio D. 1998. Molecular Cloning of Avian Matrix Gla Protein. *Biochimica et Biophysica Acta* 1395:47-49.

Witlock DR, Wyatt RD. 1978. Effects of *Eimeria tenella* Infection and Dietary Aflatoxin on Blood Coagulation of Young Broiler Chicks. *Avian Diseases* 22:481-486.

Woodhams B, Girardot O, Blanco M-J, Colesse G, Gourmelin Y. 2001. Stability of coagulation proteins in frozen plasma. *Blood Coagulation and Fibrinolysis* 12(4):229-236.

Woods B, Hagenstein R, Waldman B. 2009. News Release. Reports from Rat Island Reflect Successes and Concerns. US Fish and Wildlife Service. http://www.fws.gov/news/NewsReleases/showNews.cfm?newsId=D18536 46-F27F-23F3-9E027F384A3AF96D

Yagami K, Suh J-, Enomoto-Iwamoto M, Koyama E, Abrams WR, Shapiro IM, Pacifici M, Iwamoto M. 1999. Matrix Gla Protein is a Developmental Regulator of Chondrocyte Mineralization and, when Constitutively Expressed, Blocks Endochondral and Intramembranous Ossification in the Limb. *The Journal of Cell Biology* 147:1097-1108.

APPENDICES

Appendix A: Body weight and liver somatic indices (LSI) for Japanese quail dosed with brodifacoum groups.*

Days post-exposure	Brodifacoum Dose (mg/kg)									
	0		0.8		1.4		1.9		2.5	
	Wt (g)	LSI (%)	Wt (g)	LSI (%)	Wt (g)	LSI (%)	Wt (g)	LSI (%)	Wt (g)	LSI (%)
1	128.6 (5.1)	2.0 (0.2)	128.8 (3.0)	1.8 (0.05)	130.4 (2.8)	1.8 (0.2)	116.4 (6.5)	2.1 (0.3)	123.2 (4.2)	2.0 (0.2)
3	126.1 (6.7)	2.0 (0.1)	128.9 (4.3)	1.8 (0.08)	121.9 (4.9)	2.0 (0.2)	130.0 (5.8)	1.8 (0.2)	122.3 (2.6)	2.0 (0.1)
5	124.3 (6.6)	2.0 (0.3)	120.9 (2.2)	2.0 (0.2)	124.8 (7.8)	1.6 (0.06)	114.0 (3.0)	1.8 (0.1)	127.9 (5.0)	1.7 (0.05)
7	134.7 (7.4)	2.3 (0.3)	126.3 (3.8)	2.3 (0.4)	125.4 (3.2)	1.8 (0.4)	-	-	-	-

* Mean (SEM)

Appendix B: Summary of necropsy results for Japanese quail dosed with brodifacoum.

Dose (mg/kg)	Sex	Days Post-Exposure							
		1		**3**		**5**		**7**	
		I	**Symptoms**	**I**	**Symptoms**	**I**	**Symptoms**	**I**	**Symptoms**
0	M								
	F								
0.8	M	0 of 3		0 of 3		1 of 3	Possibly blood in intestines	1 of 1	Massive hemorrhage on left tarsus; 1 black testis
	F	1 of 3	Small bruise on right breast muscle	0 of 3		3 of 3	Hemorrhage on both feet and half of tarsus; massive hem on breast muscle; Massive cranial hem; Blood in left eye socket and mouth; Bloody 1° follicle	2 of 3	Hemorrhage on 80% of left calf, and 20% of right calf
1.4	M	1 of 3	Small bruise on leg; Minor cranial hemorrhage	2 of 3	Massive cranial hemorrhage; Hemorrhage on right elbow, both knees and breast muscle; Blood in cecum	3 of 3	Pale lungs, skull, intestines and feet; Massive hemorrhage on left calf; Large hemorrhage on left wrist; Hemorrhage under wing, on half of tarsus and calf	2 of 2	Hemorrhage on right tarsus and right shoulder joint; large hemorrhage on breast muscle with clot near heart; very white skull; massive hemorrhage around crop
	F	1 of 3	Small bruise on knee	2 of 3	Hemorrhage on right knee and thigh, left tarsus, foot, knee, under left wing and breast muscle; Possibly blood in intestines	of 3	Hemorrhage near right ear, cloaca, left wrist, half of tarsus, left calf; Pale skull; Blood in mouth and crop; Small bruises on breast muscle		
1.9	M	1 of 5	Hemorrhage at the base of gizzard; Minor bruising on both legs	0 of 3		2 of 2	Blood on back where feathers grow in; Possibly blood in testis; Bruise on left wrist; Abdominal hemorrhage; Pale skull		
	F	0 of 3		3 of 3	Full abdominal hemorrage; Extensive bleeding on back at the site of new feather growth; Pale lungs and skull; Hemorrhage on right wing and breast muscle; Possibly blood in ceca	3 of 3	Hemorrhage on breast muscle, thighs, tarsus, and head; Bruise on both wrists and base of breast muscle; Pale skull and lungs		
2.5	M	1 of 3	Hemorrhage near right testis	3 of 3	Some blood in intestines; Massive hemorrhage on right knee; Bruise at base of skull	3 of 3	Hemorrhage on calf, in shoulder socket, both biceps, at base of gizzard, on lower breast muscle, and in ceca; Full cranial hemorrhage; Pale skull and intestines		
	F	0 of 3		3 of 3	Full abdominal hemorrhage; Bleeding on back at the site of new feather growth; Blood in mouth, crop and intestines; Bloody egg follicle; Bruise on breast muscle	3 of 3	Hemorrhage on head, breast muscle, right side and shoulder, right tarsus and leg, both legs, in abdomen, cecum and mouth; Pale skull, egg follicles		

I = Incidence within group, i.e. the number of birds experiencing one of the symptoms described