BARN OWL GENETICS IN WESTERN NORTH AMERICA:

DIVERSITY, PHYLOGEOGRAPHIC STRUCTURE, CONNECTIVITY, AND POTENTIAL FOR A GENETIC BASIS TO ANTICOAGULANT RODENTICIDE SUSCEPTIBILITY

by

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ABSTRACT

The barn owl (*Tyto alba*) is distributed across much of North America in areas with extensive old-field and grassland habitat. Barn owls are threatened in British Columbia (BC), where the population has declined by 50% in the last 3 decades. I investigated the genetic diversity and phylogeographic patterns of barn owls in western North America, ranging from BC to California, and one eastern population from Pennsylvania. Using 8 polymorphic microsatellite markers (N=126) and ND2 mitochondrial sequences (N=37), I found a high degree of gene flow among the continental sampled regions (global F_{ST} = 0.028). The BC mainland population, despite its northwestern geographic peripheral location and ongoing habitat degradation, is not genetically depauperate. However, individuals from Vancouver Island exhibited the lowest genetic diversity of all sampled locations, likely as a result of its insular nature. The low global F_{ST} value estimated from this study suggests that their habitat is well connected across North America. Additionally, microsatellite data revealed that the Santa Barbara Island population showed genetic divergence from its continental counterpart. Mitochondrial data, however, demonstrated that this island population is not monophyletic, and thus cannot be designated as an Evolutionarily Significant Unit.

Anticoagulant rodenticides (ARs) are pesticides widely employed worldwide to reduce rodent infestations. Avian predators that hunt extensively for small rodents are at risk of secondary poisoning. AR causes internal bleeding by disrupting the Vitamin K cycle, which is essential for blood clotting. Tolerance to AR appears to be highly variable among individuals for any given avian species. I examined whether single point mutations in the CYP2C45 gene are associated with increased or decreased susceptibility to AR in barn owls. I identified a position that showed a heterozygous C/T in one particular individual with low tolerance, whereas all other individuals exhibited a homozygous C. This transversion results in an amino acid substitution from alanine to valine at a conserved region that could potentially have deleterious effects on the function of and structure the protein. However, it is also possible that the CYP2C45 enzyme was not severely affected due to this amino acid change since both alanine and valine are non-polar/hydrophobic.

PREFACE

This research was conducted in collaboration with my supervisors, Dr. John Elliott and Dr. Kathy Martin, who contributed to the design of the experiment. I generated all of the genetics data at the Genetics Data Centre (GDC) with guidance from Dr. Carol Ritland. I also conducted all of the statistical analyses. Barn owl post-mortem and anticoagulant rodenticide residue data were provided by Dr. John Elliott (Environment Canada).

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CHAPTER 1 – GENERAL INTRODUCTION AND THESIS OVERVIEW

1.1. Implementation of genetics in conservation biology

Human-caused threats leading to declines in many avian populations are wide-ranging across the globe. Agricultural intensification is assigned as the factor responsible for the decline of 87% of the threatened bird species, followed by extensive forest harvest and the spread of residential/commercial development into wildlife habitat (BirdLife International 2008). Such anthropogenic stressors collectively lead to the destruction and degradation of wildlife habitat, forcing dramatic range contraction in many wildlife populations, including birds. Moreover, human development can also cause habitat fragmentation, which reduces the degree of connectivity between wildlife populations. These sparsely distributed populations as a result of multiple anthropogenic factors can become isolated, which ultimately increases the chance of local extirpation (Levins 1969). Other more direct causes of wildlife mortality that contribute to the overall population decline include road-kills, illegal hunting and exploitation, and invasive species (IUCN 2009).

The implementation of genetic approaches is critical in the field of conservation biology and wildlife management (Sarre and Georges, 2009). With the advances in genetic technologies and the development of hypervariable DNA markers, the genetic profile of organisms can now be determined efficiently at the individual level, allowing biologists to gain insights into their population structure and connectivity, mating systems, natural barriers, and epizootics.

For instance, mitochondrial DNA (mtDNA) is particularly useful for the identification of species from degraded samples, such as fecal materials (Sugimoto et al. 2006, Wasser and Hunt 2005) and old museum specimens in the form of feathers, bones or eggs (Hsieh et al. 2006). When compared to the nuclear genome, there are typically higher copies of mitochondria found per cell, and therefore a higher chance of successfully extracting intact mtDNA from degraded samples. As such, mtDNA has been proved to have extensive application in wildlife conservation and management, including identifying genetically distinct units due to historical

divergence (mountain chickadees [*Poecile gambeli*], Spellman et al. 2007), determining historic population bottlenecks (northern spotted owls [*Strix occidentalis caurina*], Funk et al. 2008), and even assessing mammal diversity from fly-derived DNA (Calvignac-Spencer et al. 2013).

Microsatellite markers, on the other hand, are nuclear markers comprised of tandem repeats of 1-6 base pairs motifs. Being highly variable sequences and typically selectively neutral, they are widely used to interpret parentage and relatedness, population structure, genetic diversity, and dispersal rates (Sarre and Georges, 2009). Unlike mitochondrial DNA which is inherited solely through the female line, microsatellites alleles are inherited from both parents and evolve at different rates depending on the level of polymorphism. As a result, microsatellite data are often used in conjunction with mtDNA data in many wildlife population and phylogenetics studies (e.g. Hull et al. 2010, Oyler-McCance et al. 1999). Using both markers provides two independent perspectives on the patterns of genetic variation, and higher resolution to infer the evolution and demographic history of the species of interest.

While molecular population genetics has traditionally been more closely associated with ecology and evolutionary biology, it has also recently been implemented in ecotoxicology. Genetic ecotoxicology is a young but emerging field studying the linkage between chemical pollutants exposure and the genetics of wildlife populations (Bickham 2011; Guertin et al. 2012). Chronic exposure to contaminants can negatively influence individual reproductive success, which can lead to an overall decline in population size. Genetic drift is often associated with a loss of genetic diversity in a small population. In this context, many studies in genetic ecotoxicology aim to investigate whether contaminated populations are genetically depauperate by using neutral genetic markers (Bickham 2011).

Few studies, however, have examined whether certain pollutants have altered the genotype frequencies of a population using functional genes. Exposure to toxicants can potentially lead to the selection of certain survivorship loci, resulting in changes in allelic or genotypic frequencies within a population (Bickham 2011). For instance, cotton rats (*Sigmodon hispidus*) inhabiting oil-contaminated sites showed reduced genetic variability in the major

histocompatibility genes (MHC), a family of polymorphic genes involved in immune function (Pfau et al. 2001). However, the identification of adaptive functional genes associated with contaminant exposure is in fact difficult to accomplish. Instead, most studies have only provided indirect evidence, such as tracking the persistence of pollutant resistance in multiple generations, and identifying increased frequency of certain contaminant indicative RAPD (Random Amplified Polymorphic DNA) bands (Belfiore & Andereson 2001). In order to demonstrate direct, concrete evidence of genetic adaption to pollutants, genes that become activated as a bodily response to the xenobiotic substance must first be identified, followed by investigating the potential linkage between certain nucleotide changes and increased resistance or susceptibility.

The first objective of my study was to investigate the genetic diversity and phylogeographic patterns of barn owls populations in western North America, ranging from British Columbia (BC) to southern California, and one eastern population from Pennsylvania. Specifically, I assessed the genetic diversity of barn owls in BC, a declining peripheral population currently experiencing habitat degradation within much of their range. I also aimed to investigate the degree of gene flow among the sampled populations, and to identify any potential geographic barriers that may have given rise to genetically distinct populations. The second objective of this study was to conduct preliminary investigations of the genetic basis behind differential rodenticide sensitivity in barn owls. I examined whether any potential SNPs (single nucleotide polymorphism) from the avian CYP2C45 gene – part of the cytochrome P450 gene family responsible for metabolizing xenobiotics in vertebrates – are potentially associated with varying levels of tolerance to rodenticide.

1.2. Study species – Barn owls (*Tyto alba*)

The barn owl (*Tyto alba*) is one of the most widely distributed owls globally, found on every continent except Antarctica (Marti et al. 2005). There are currently 32 described subspecies worldwide; *Tyto alba pratincola* is the subspecies found on mainland North America and most parts of central America (Marti et al. 2005, Figure 1.1). Although barn owls are a medium-sized

owl, their body mass varies greatly depending on the region: males range from 400 to 560g, and females 420-800g.

Prior to human settlement, barn owls nested in tree cavities, underground burrows, and abandoned nests of other bird species. With the expansion of human agriculture, barn owls have exploited man-made structures extensively for nesting and roosting, including barn lofts and crevices in abandoned buildings (Taylor 1994). As a nocturnal hunter, barn owls fly about 1.5 – 4.5m above the ground in open habitats preying mostly on small terrestrial rodents (74-100% of diet). Songbirds, reptiles, amphibians, and arthropods make up a smaller portion of their diet (Taylor 1994). In North America, voles (*Microtus*) are the dominant prey; these small rodents are found in high densities in open grassland or old-field habitats (Marti et al. 2005). Given their preferred habitat usage for foraging and nesting, barn owls have long been closely associated with human settlements, in particular traditional farmlands with barns, sheds, and dense grass pastures.

1.3. Study area – Western North America

In western North America, barn owls are distributed from the southwestern corner of British Columbia to Washington, Oregon, and California (Figure 1.1). Within their western range, they are most prevalent in agricultural regions, ranchlands, suburban residential areas, and grasslands with scattered trees (Marti et al. 2005). In comparison, areas with extensive snow cover and high elevations are uninhabitable for them. Barn owls are highly vagile, able to disperse distances of up to 1267 km from their natal site (Marti 1999). This variability in fledgling dispersal distance is attributed to annual fluctuations in the abundance of voles (Taylor 1994). During years of low vole abundance, young owls are forced to travel further from their natal site to find areas with ample food supply. When vole populations are high, however, owls would not have to disperse great distances in search of suitable foraging habitat.

Barn owls are listed as “apparently secure” in the States of Washington and Oregon (Natureserve, 2014). Although their conservation status is unranked in California, barn owls

are known to nest in large numbers throughout most of the state, including most of the Channel Island archipelago (Marti et al. 2005).

While the present distribution in North America spans much of Mexico and the USA, barn owls occur in only two provinces in Canada: Ontario and British Columbia (BC). The Ontario population is believed to be well under a minimum viable population level (COSEWIC 2010) given that only 5-10 breeding pairs remain. In comparison, the western population is confined to the southwestern corner of BC (Figure 1.1), with the most recent estimate to be no more than 500 mature individuals (COSEWIC 2010). Barn owls in BC cannot disperse beyond their current range due to their limited cold tolerance and inability to hunt through snow cover. As a result, they are considered as the most northern distribution in North America (Marti et al. 2005).

The western population in Canada is predominately found in BC's Lower Mainland (including the Fraser Valley) and southeastern Vancouver Island. Since field voles (*Microtus townsendi*) make up a large portion of their diet in BC, barn owls rely heavily on extensive foraging habitats, including open agricultural landscapes and rough pastures (Taylor 1994). Furthermore, sheltered cavity sites, such as artificially installed nest boxes in old wooden barns and grasslands, are equally important for nesting and roosting (Taylor 1994). However, southwestern BC includes some of the fastest growing human communities in Canada, meaning there is continuing pressure to convert their remaining agricultural and grassland habitat into urban and industrial developments. Hindmarch et al. (2012) estimated that from 1993-2008 in the Delta and Surrey area of the Fraser Valley, both suitable nest sites in barns and grassland cover had reduced by about 53% and 30%, respectively, whereas urban development has increased by 133%.

In addition to habitat loss, barn owls in BC are particularly susceptible to vehicle-induced mortality due to their habit of relatively slow and low flight. Andrusiak (1994) reported that road kills were assigned as the cause of death for 63% of the 341 carcasses examined across BC. Nest sites with high traffic exposure are less likely occupied by barn owls (Hindmarch et al.

2012). Traffic volume in the Fraser Valley has already increased by 33% since the early 1990's (Hindmarch et al. 2012). With this ongoing trend, the distribution of barn owls has been becoming increasingly restricted by the expanding road networks in BC.

The combined effects of these anthropogenic stressors have resulted in the decline of the BC barn owl population from approximately 1000 mature individuals in 1983 to 250-500 in 2008 (Campbell and Campbell 1983, COSEWIC 2010); consequently, they have recently been uplisted to *Threatened* by Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2010). There is a growing concern that with this current trend, barn owls may soon be extirpated in Canada.

1.4. Secondary poisoning from rodenticide

Second generation anticoagulant rodenticides (SGARs) are pesticides widely employed in urban and rural farmlands worldwide to reduce rodent infestations. These toxicants were first introduced in the 1970's in response to the increasing incidence of rat populations resistant to first generation anticoagulant rodenticide (FGAR) compounds, in particular warfarin (Buckle et al. 1994). Both warfarin and SGARs are coumarin derivative compounds. They disrupt the regeneration and recycling of vitamin K, a key component required for blood coagulation. Consequently, ingestion of these rodenticides leads to effects on the functioning of vitamin K clotting factors essential to normal blood coagulation, which at lethal doses causes internal hemorrhage and death (Pelz and Kohn 2011).

SGARs are in general more acutely toxic than FGARs. In contrast to warfarin where multiple doses are required to exert a lethal effect, SGARs provide a fatal dose after only a single ingestion of bait (Albert et al. 2010) and have relatively longer half-lives (Vandenbroucke et al. 2008). However, the toxic effect of SGAR is not immediate, meaning the poisoned rodents will typically stay alive for several days and continue to feed on the bait (Cox and Smith 1992); as a result, the rodenticide concentration in their bodies will more likely exceed the LD50, or even LD100 dose. Moreover, poisoned rodents may remain in a state of lethargy, or even

motionless in open areas, making them more predisposed to consumption to predators or scavengers (Cox and Smith 1992). Consequently, predators, particularly birds of prey, that hunt extensively for small rodents are at risk of secondary poisoning. In a study where four species of birds of prey were tested for rodenticide residue, including red-tailed hawks (*Buteo jamaicensis*), barred owls (*Strix varia*), eastern screech owls (*Megascops asio*) and great horned owls (*Bubo virginianus*), 86% had either FGAR or SGAR residues in their liver tissues, where brodifacoum was identified to be the most prevalent anticoagulant rodenticide (AR) compound (99%) in the positive birds (Murray 2011). This demonstrates that AR chemicals have accumulated up the food chain and are widely found in various avian predators.

In the Lower Mainland, British Columbia, there has been a significant increase in the sales of rodenticide from 1995 to 2009, with two SGARs (brodifacoum and bromadiolone) showing the highest sales (Elliott et al. 2014). This increase in usage of SGARs is causing high rates of mortality in BC barn owls. While rodenticide was the direct cause of death for 3% of the 78 barn owl carcasses examined in BC, 62% of dead owls contained residues of one or more SGAR compounds in the liver (Albert et al. 2010). It is widely speculated that having such sublethal dose of anticoagulant rodenticides, although not fatal, could reduce their hunting efficiency and nesting success (Taylor 1994), and may cause prolonged clotting times increasing the health and survival consequences from even minor trauma (Rattner et al. 2014).

Newton et al. (1998) suggested that 0.1 mg/kg of SGAR residues in liver is associated with increased probability of poisoning risk. This proposed value was based on a necropsy examination where almost all the barn owls having died from rodenticide poisoning had liver concentrations > 0.1 mg/kg. However, a toxicity threshold value for barn owls is in fact difficult to determine, as SGAR concentrations associated with toxicosis symptoms vary markedly amongst individual birds. The barn owls experimentally poisoned with rodenticide had liver residues in the range of 0.2-1.72 mg/kg (Newton et al. 1999), suggesting almost an order of magnitude difference in susceptibility to these anticoagulants. In another post-mortem examination of barn owls in the United Kingdom, 9 of the 10 carcasses that had 0.100 – 0.337 mg/kg of SGAR residue in their livers did not show signs of hemorrhaging due to secondary

poisoning (Walker et al. 2013). In contrast, other carcasses, which had SGAR concentration as low as 0.060 mg/g, were diagnosed with secondary poisoning as the cause of death (i.e. evidence of hemorrhaging not associated with physical trauma). In another owl species, the eastern screech owls (*Megascops asio*), both liver residue and the time taken for a blood clot to form (i.e. prothrombin time) were likewise highly variable upon oral exposure to diphacinone (Rattner et al. 2014). Tolerance to rodenticide is evidently highly variable among individual barn owls; however, currently there is very little understanding of whether there might be a genetic basis for this phenomenon in avian species.

1.5. Thesis overview

Antoniazza et al. (2009) found that barn owls in continental Europe exhibited very low genetic structure and high levels of gene flow, and attributed these results to their ability to disperse long distances. Australian masked owls (*Tyto novaehollandiae*), an owl species closely related to barn owls, also showed no genetic difference across continental Australia (Hogan et al. 2013). However, masked owls from two Australian islands – Lord Howe Island and Tasmania – were found to be genetically distinct as a result of restricted gene flow from the mainland population. In Chapter 2, I used 8 polymorphic microsatellite markers (N = 126 individuals) and amplified a 950bp segment of mitochondrial NAD2 gene (N = 37 individuals) to investigate the phylogeographic structure of barn owls in western North America. In addition to sampling individuals from 4 continental locations (BC mainland, west Oregon, east Oregon, south California) and 2 island populations (Vancouver Island and Santa Barbara Island) in western North America, I also included a northeastern population (Pennsylvania). Using the software program STRUCTURE, a Bayesian model-based algorithm, my results showed that the Santa Barbara Island is genetically distinct. In comparison, the other 6 sampling sites showed a high degree of gene flow, suggesting that grassland and old-field habitats are well connected across their range.

McLarty (1995) used VNTRs (Variable Number Tandem Repeats) to show that the BC mainland barn owl population has less genetic variation when compared to the Utah and

California populations. However, using this DNA fingerprinting approach is not as reliable as the more multivariable and codominant microsatellite for assessing genetic diversity. Therefore, further investigation is required to evaluate the level of genetic diversity in the BC barn owl population. In Chapter 2, I used 8 polymorphic microsatellite markers to examine the genetic diversity of two declining barn owl populations in BC – BC mainland and Vancouver Island – when compared to other more stable and robust populations. My results showed that the BC mainland individuals are not genetically depauperate despite its northwestern geographic peripheral location and ongoing habitat degradation. However, individuals from Vancouver Island exhibited the lowest genetic diversity of all sampled locations, likely attributed to the combined effects of its peripheral location and insular nature.

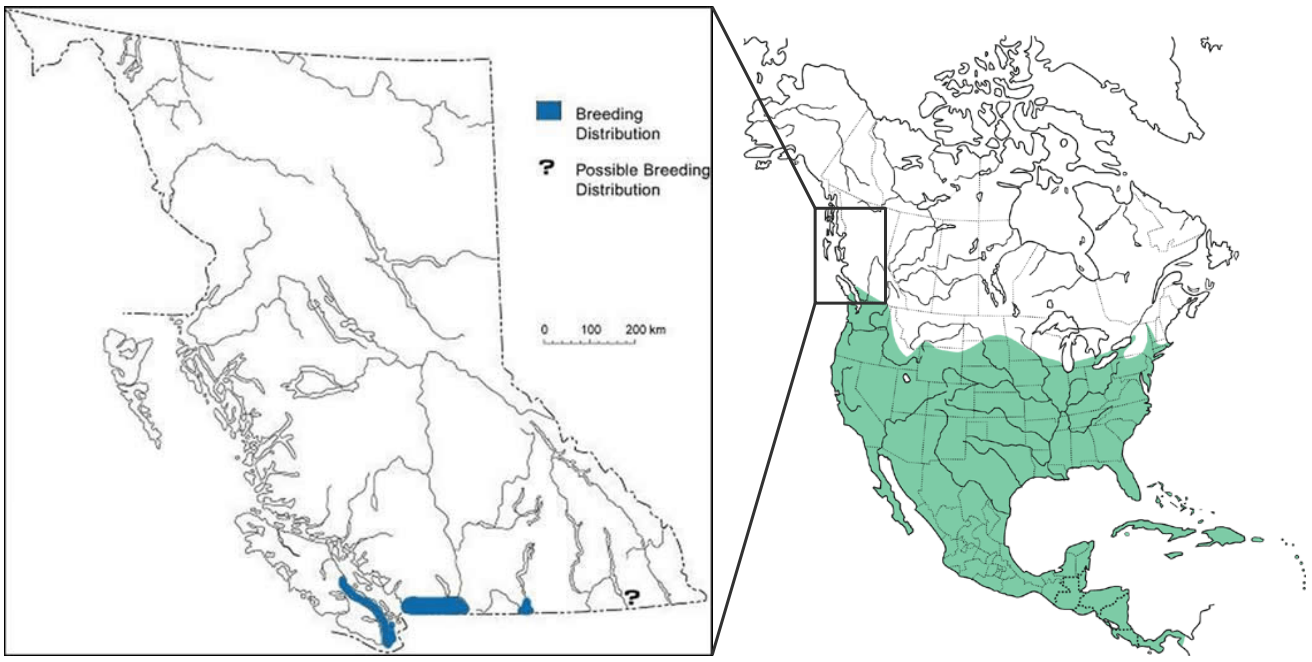


Figure 1.1 – Right: distribution of Barn Owls (*Tyto alba*) in North America (Marti 2005). Left: distribution of Barn Owls in British Columbia (COSEWIC 2010).

CHAPTER 2 – BARN OWLS IN NORTH AMERICA: PHYLOGEOGRAPHIC STRUCTURE, CONNECTIVITY, AND GENETIC DIVERSITY

2.1. Introduction

Investigations of regional genetic differentiation are crucial for determining patterns in population structure and informing management efforts for species of conservation concern. By identifying the potential barriers that determine their contemporary geographic distribution, conservation managers will be able to understand the pattern and rate of dispersal of the animal. In addition, it will also provide the necessary evolutionary and ecological framework to properly manage populations with reduced gene flow or genetic diversity.

Populations with restricted gene flow from a source population can exhibit divergence in their genetic signatures. When gene flow becomes virtually absent, especially under high selection pressure and low population size, the isolated population can develop reciprocally monophyletic alleles and contain unique haplotypes (Birky et al. 1989). Often designated as an Evolutionarily Significant Unit (ESU), these genetically distinct populations can evolve independently from their conspecifics, and should warrant additional conservation effort (Moritz 1994). On the other hand, a population is classified as a Management Unit (MU) when it has diverged in allele frequency, yet is not a monophyletic lineage. In other words, as a result of the low levels of gene flow, this population is “functionally independent” and relies on local birth and death rates rather than immigration to sustain itself (Moritz 1994). Island populations tend to exhibit genetic differentiation from their mainland counterparts, and thus are often designated with ESUs or MUs depending on whether reciprocal monophyly has been achieved. At the same time, however, the insular nature of these populations is often fraught with reduced genetic diversity due to insufficient gene flow from source populations (Frankham 1997).

Low genetic diversity is not only associated with isolated islands or regions, but also populations at the edge of their distribution range. Compared to those at the core of the

distribution, populations at the periphery tend to reside in a less suitable habitat and hence have lower densities with lower migration rates and restricted gene flow (Hoffman and Blows 1994). Peripheral populations, as a result, generally exhibit lower genetic diversity. Habitat removal and fragmentation can similarly induce drift and loss of alleles (Frankel and Soule 1981). Both of these phenomena have been observed in numerous animals, including the greater prairie chicken (*Tympanuchus cupido*, Bouzat et al. 2008), black grouse (*Tetrao tetrix*, Caizergues et al. 2003), fishers (*Martes pennant*, Wisely et al. 2004), and wolverines (*Gulo gulo*, Cegelski et al. 2003). Other studies, to the contrary, have demonstrated that this concept cannot be applied to all species (Eckert et al. 2008). For instance, the population of greater sage grouse (*Centrocercus urophasianus*) living in a fragmented and northern peripheral habitat was not genetically depauperate (Bush et al. 2011). The authors speculated that despite the grouse being relatively sedentary birds, occasional long distance dispersers allow for sufficient gene flow among populations. Occasional dispersal, coupled with the close proximity between the core and peripheral populations, has maintained the high genetic diversity in the peripheral and fragmented regions.

Barn owls (*Tyto alba*) are one of the most globally widespread bird species found on all continents except for Antarctica. These owls have adapted to living in close proximity to humans, and in addition to nesting in natural cavities in trees and cliff sides, they also make use of man-made structures such as traditional wooden barns and abandoned buildings. Further, their main prey, the field vole (*Microtus townsendi*), is found in high densities in grassland fields (Marti et al. 2005). Given their high adaptability to humanized landscapes, barn owls are commonly found throughout most of the USA with no apparent biogeographic disjunction in their current distribution in North America (Figure 1, Marti et al. 2005). However, natural boundaries not perceived on a species distribution map can still be present, giving rise to population structuring at a regional scale. Numerous cases of genetic isolation and segregation have been documented for avian species residing in western North America due to its heterogeneous landscape. For instance, the Cascade Range acts as a geographic barrier for gene flow, resulting in genetically distinct clades on either side of the mountains (e.g. Manthey et al. 2011, Walstrom et al. 2011, Rush et al. 2009). Moreover, isolation by distance

has also been shown in several other species (e.g. Barrowclough et al. 2011, Hull et al. 2010). Currently, no studies have investigated the population structuring pattern of barn owls in North America, whether genetic differentiation exists across its breeding range, or if it is one large panmictic population.

Santa Barbara Island is the smallest island of the Channel Islands archipelago. Approximately 5-25 resident barn owls breed on the island, preying on deer mice (*Peromyscus maniculatus*) and small seabirds (Fellers and Drost 1991). These owls have been suggested to be partially responsible for the decline of a nesting population of Scripps's Murrelet (*Synthliboramphus scrippsi*) on Santa Barbara Island as adult predation contributes to approximately 30% of all annual murrelet mortality (Nur et al. 2013). Relocating barn owls to mainland California has been suggested as a potential solution to increase murrelet numbers (Millus et al. 2007, Nur et al. 2013, S. Thomsen pers. comm.). However, such management action can result in the removal of a potential ESU of Santa Barbara Island barn owls given the population's insular nature. Moreover, translocation efforts may be futile if dispersal from mainland California to Santa Barbara Island is frequent enough to re-establish another barn owl population by filling the empty niche. Therefore, before any management actions proceed, these two concerns should be addressed.

In contrast to the more stable populations in the USA, the only existing viable population in Canada is confined to the southwestern corner of British Columbia (BC), more specifically the Lower Mainland (including the Fraser Valley), southeastern Vancouver Island, and South Okanagan (Figure 1, COSEWIC 2010). Due to their limited cold tolerance and inability to hunt through snow cover, barn owls in BC cannot disperse to the north or east beyond their current range, making the population the most northerly distributed in North America (Marti et al. 2005). Currently listed as *Threatened* federally, this population has declined drastically in the past 30 years primarily as a result of habitat degradation (COSEWIC 2010). Southwestern BC includes some of the fastest growing human communities in Canada, meaning there is an ongoing pressure to convert remaining agricultural and grassland habitat into urban and industrial developments. Hindmarch et al. (2012) estimated that from 1993-2008 in the Delta and Surrey

area of the Lower Mainland, suitable nest sites in barns and grassland cover were reduced by 53% and 30%, respectively, whereas urban development increased by 133%. It was estimated that the BC barn owl population dropped from approximately 1000 mature individuals in 1983 (Campbell and Campbell 1983) to less than 500 in 2008 (COSEWIC 2010). Concerns regarding the compromise of its genetic diversity due to diminishing numbers should be addressed as part of the population's recovery strategy.

In this study, I used microsatellite genetic markers as well as mitochondrial sequence data to assess the population genetic structure of barn owls in North America. Specifically, the objectives of this study were to (1) determine if the Cascade Range acts as a natural barrier that has given rise to two genetically divergent populations on either side of the mountain ranges; (2) investigate whether barn owls are exhibiting isolation by distance, i.e. whether the BC population (most northwestern range) is genetically less similar to the Southern California (southwestern range) and Pennsylvania (northeastern range) populations when compared to its neighbouring population in Western Oregon; (3) assess whether the Santa Barbara Island barn owls are genetically distinct from their mainland counterpart; and finally (4) examine evidence for possible reduction of genetic diversity in the BC population, given its peripheral location and ongoing habitat degradation.

2.2. Methods

Population sampling

I collected whole blood, toe pads, contour feathers, or muscle tissue samples from 126 individual barn owls from seven general regions in North America (Figure 2.1). All samples were collected within a 3-year period (2011-2013) from surveys of previously known nesting and roosting sites, wildlife rehabilitation facilities, or road kills.

I sampled mostly nestlings to ensure that individuals accurately represented their associated geographic regions, as barn owls are known to disperse over several hundred kilometers

(Marti 1999, Paradis et al. 1998). However, most samples from Santa Barbara Island and Vancouver Island were collected via trapping the adults or salvaging carcasses due to inaccessible or limited nest sites in these two study areas. I avoided collecting more than one individual per nest site to minimize bias generated from sibling relatedness. All samples were stored in 100% EtOH. From each of the 126 samples, I extracted total DNA from muscle (5mg) and blood (10 ul) using a modified protocol of Meulenbelt et al. (1995), and from toe pad (5mg) and feathers using QiAamp DNA Investigator Kit (QIAGEN, Inc.). For feather samples, I only genotyped those with good DNA quality (confirmed using Nano-drop) to avoid producing incorrect genotypes (Hogan et al. 2008).

Microsatellite data collection and analysis

I genotyped all 126 individuals using eight polymorphic microsatellite loci from Burri et al. (2008). PCRs were performed in a final volume of 10μL containing 5-15ng of template DNA, 1xPCR buffer, 0.2μM of dNTPs, 0.3 U of *Taq*, 0.1μM of each forward and reverse primer, and 0.05μM of IR40-labeled M13 primer (LICOR Inc.). Table 2.1 shows the gene diversity and amplification results at the eight loci. Samples were analyzed on 6% polyacrylamide gels on a LICOR 4200 automated sequencer (LICOR Inc.). Alleles were scored by comparison with molecular weight standards using the SAGA Generation 2 software (LICOR Inc.).

I tested the dataset for deviation from Hardy-Weinberg equilibrium and linkage equilibrium using Genepop 4.0 (Rousset 2008). I also tested for evidence of null (i.e. unamplified) alleles, scoring errors, and large allele dropouts using the program Micro-checker version 2.2.3 (van Oosterhout et al. 2004). Using GenoDive 2.0 (Meirmans and Van Tienderen 2004), I calculated standard measures for assessing genetic diversity: observed heterozygosity (H_O), expected heterozygosity (H_E), and allelic richness (AR_C). Allelic richness were corrected for unequal sample sizes using the HP-Rare software (Kalinowski 2005). I tested for differences in H_O, H_E, and AR_C between the sampling locations using a Wilcoxon signed rank test, which pairs the data by locus. To assess whether barn owls in the northwestern periphery (i.e. the BC populations) exhibit lower genetic diversity, 6 samples from Washington mainland were

excluded when calculating the measures of genetic diversity. To evaluate the genetic distances between the sampling locations, I calculated pairwise population differentiation estimates (F_{ST}) using GenoDive 2.0 (Meirmans and Van Tienderen 2004). The pairwise F_{ST} estimates were generated from 50,000 permutations with the False Discovery Rate (FDR) correction (Benjamini and Hochberg 1995) applied to the p-values.

Population structure was inferred by using a Bayesian model-based clustering method with STRUCTURE version 2.3 (Pritchard et al. 2000). STRUCTURE uses genotypic data to determine the number of distinct genetic clusters (*K*) among the sample locations, and estimates individual assignment probability to each resolved cluster. Due to low overall genetic structuring (global F_{ST} = 0.028), STRUCTURE was run using an admixture model including a location prior as suggested by Hubisz et al. (2009). Twenty replicate runs (100,000 Markov Chain Monte Carlo step burn-in plus an additional 500,000 runs) were performed for each value of K. Results were summarized using STRUCTURE Harvester version 0.6.6 (Earl and vonHoldt 2012), which generated a plot of the mean value of L(K) (ln likelihood of data) at each *K*. I then inferred the most likely number of clusters by identifying the highest L(K) value with relatively small variance.

To further investigate which sampled regions were the most distinct, I used a Monte Carlo test (GenoDive 2.0, Meirmans and Van Tienderen 2004) to assign individuals to the seven sampled regions by calculating the likelihood that the individual's genotype is found in a region based on each region's allele frequencies (Paetkau et al. 1995). I replaced any allele frequencies that were found to be equal to zero in a certain region with a fixed, low allele frequency (0.005); this was to take into the account that an allele may in reality be present in a region at a low frequency, but was not sampled (Paetkau et al. 2004). I selected 0.002 as the significance threshold (alpha-level) for the Monte Carlo test, as suggested by Paetkau et al. (2004). 10,000 datasets were given to simulate the Monte Carlo test to produce a null-distribution of likelihood values with which the values for the sampled individuals were compared.

Mitochondrial data collection and analysis

I amplified a segment of the NAD2 mitochondrial region (1010 base pairs) using primers L5219 and H6313 (Sorenson et al. 1999). I chose barn owl individuals from four sampling locations within the barn owl's range in North America: BC representing the northwest (N=9), southern California representing the southwest (N=8 + 1 from Genbank, accession #: EU601052.1), Pennsylvania representing the northeast (N=8), and Santa Barbara Island representing an insular population (N=10). I selected two Australian barn owls (*Tyto alba delicatula*, accession #: EU166976.1 and EU410491.1) as the outgroup since the two subspecies share a recent common ancestor with a short branch length between them (Wink et al. 2009). PCR products were sequenced at NAPS Unit, University of British Columbia, using Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Ontario, Canada) and were resolved on Applied Biosystems 3730S 48-capillary DNA analyzer. I edited the chromatograms using Sequencher Demo version (Gene Codes Corporation).

I aligned all 37 sequences with MUSCLE (Edgar 2004), and used DNASP 5.0 (Rozas et al. 2003) to calculate the haplotype diversity for each sampled region, as well as the extent of genetic differentiation between them based on the haplotype statistic, H_{ST} (Hudson et al. 1992). Statistical significance of the pairwise H_{ST} values was based on chi-square tests with a FDR correction (Benjamin and Hochberg 1995). Phylogenetic relationships among haplotypes were visualized by constructing a median-joining network (Bandelt et al. 1999) using the program PopART version 1.5.1 (Leigh 2014).

2.3. Results

Microsatellite Data

All 8 microsatellite loci conformed to Hardy-Weinberg equilibrium, and I detected no linkage disequilibrium. Similarly, I found no evidence for the presence of scoring errors, large allele dropouts, or null alleles, with the exception of locus TA413 where MicroChecker detected an

excess of homozygotes at allele size classes 203 and 211, suggesting the presence of null alleles. However, I identified 5 and 3 homozygous individuals at the 203 and 211 class sizes respectively, all of which were from Santa Barbara Island. I also confirmed that no alleles were mis-scored or unamplified for these 8 samples. After running the analysis again without the 8 homozygous barn owl individuals from Santa Barbara Island, excess homozygotes were no longer detected at the TA413 locus.

Pairwise F_{ST} differentiations between Santa Barbara Island and all other sampling regions were statistically significant after a FDR correction (Table 2.2). The highest F_{ST} estimate was with Pennsylvania (0.08), whereas BC/WA showed the lowest (0.05). In contrast, all pairwise comparisons with Pennsylvania were relatively smaller, ranging from 0.02 – 0.03 (with the exception of Santa Barbara Island). These F_{ST} values were significant below the 0.05 alpha level, but comparisons to Vancouver Island and West Oregon were not significant after a FDR correction. Furthermore, a low F_{ST} value was found between BC/WA and Vancouver Island (0.02) and was significant after a FDR correction. All other pairwise F_{ST} differentiations were not significant, including between western Oregon and eastern Oregon.

Bayesian clustering results from STRUCTURE unequivocally revealed that the likelihood of the number of populations (L(K)) was the highest at K = 2 (Figure 2.2a). After excluding the Santa Barbara Island individuals, however, the highest L(K) value became K = 1 (Figure 2.2b), suggesting that no population structuring signal was detected when data was analyzed without the Santa Barbara Island population. Strong contemporary genetic structure was evident in the Q-value (admixture coefficient) plot where individuals were clustered into two distinct groups: Santa Barbara Island in blue, and all other regions in red (Figure 2.3). All individuals in these two groups exhibited high Q-values associated with their own clustering, with the exception of two individuals from Santa Barbara Island which showed relatively higher proportion of red than blue. These two “interlopers” can be considered as recent migrants from a population genetics point of view.

Further analysis based on individual assignment showed that 89.5% of the Santa Barbara Island individuals were properly inferred to their region of origin (i.e. Santa Barbara Island), and only 10.6% (2 individuals) were assigned to other regions (Table 2.3); these two individuals corresponded to the two migrants detected in Figure 2.3. In contrast, the assignment of individuals from the other six sampled regions was more or less evenly distributed amongst all the inferred regions. For example, 30.8% (N=4) of the 13 genotyped barn owls from Pennsylvania were inferred as from BC/WA, 7.7% (N=1) from West Oregon, 23.1% (N=3) from Southern California, and 38.5% (N=5) from their place of origin (i.e. Pennsylvania).

Mitochondrial data

Results from a median-joining haplotype network showed that there is a lack of phylogeographic structure among the four sampled regions: BC, California, Santa Barbara Island, and Pennsylvania (Figure 2.4). In other words, there was no apparent association between haplotype and any geographic location. Santa Barbara individuals did not contain unique haplotypes, but rather shared the same ones with all 3 other sampled regions. Moreover, Table 4 also shows that all pairwise H_{ST} comparisons were not significant, with the exception of Santa Barbara Island versus BC (0.17) and Pennsylvania (0.16).

Microsatellite & mitochondrial data – genetic diversity

There was no evidence of low genetic diversity for the BC mainland as allelic richness (AR_C), expected heterozygosity (H_E), observed heterzygosity (H_O), and haplotype diversity (Hd) were all relatively high compared to other non-island regions (Table 4). Comparatively, Vancouver Island exhibited relatively lower values for all three estimates of genetic diversity: AR_C (3.71), H_O (0.64), and H_E (0.66). Wilcoxon signed rank test showed that all three estimates are significantly different from BC mainland (AR_C: W(7) = 2, -2.240, p = 0.025; H_E: W(7) = 0, -2.201, p = 0.028; H_O: W(7) = 0, -2.028, p = 0.043). AR_C and H_E between Vancouver Island and East Oregon also differed significantly (W(7) = 0, -2.521, p = 0.012; W(7) = 1, -2.380, p = 0.017, respectively). Santa Barbara Island likewise displayed evidence of low genetic diversity due to

its low AR_C value (3.56); however, this was not significant when compared to other sampling regions. Its Hd was also the lowest of all sampled regions (0.47) as only 2 haplotypes were found from the 10 individuals analyzed.

2.4. Discussion

Gene flow across continental North America

Both nuclear and mitochondrial data showed that there is a high degree of gene flow among the sampled sites within the breeding range of barn owls (*Tyto alba*) in North America other than a distinct population found on Santa Barbara Island. I found no evidence of isolation by distance along its western distribution, and that there is overall little to no genetic structure among the western continental populations. Barn owl habitat is often associated with agricultural and grassland landscapes (Taylor 1994), whereas areas with long-term snow cover and high mountain elevations are unsuitable for them. However, contrary to what one would expect, the Cascade Range does not appear to function as a barrier for gene flow between the two populations on either side of the ranges. Western Oregon and Eastern Oregon individuals are genetically similar to each other as shown by my microsatellite data. Furthermore, there seems to be regular effective migration along their western breeding range, even between southern California and British Columbia (>1900 km apart) as indicated by the rather low F_{ST} value. In comparison, although my findings detected a slightly stronger signal in allele frequency divergence between western and Pennsylvania populations, the F_{ST} values were still considerably low given the locations (approximately 3400 km). These results suggest that grassland and old-field agricultural habitats for barn owls are generally well connected and homogeneous in continental North America, allowing sufficient gene flow throughout their range. While I recognize that there is a sampling gap in central North America, the small genetic distance between Pennsylvania and the western populations detected in this study is indicative of high levels of gene flow within their North American distribution.

The high connectivity between my sampled regions can also be attributed to the ability of barn owls to travel long distances. Based on band recovery data, the average dispersal distance from natal sites was 102.9 km, and ranged from 0 to 1267 km (Marti 1999). Fledgling dispersal distance is particularly greater in years of low vole abundance, as fledglings need to travel further in search of food (Taylor 1999). While 88% of band recoveries in the southern USA were within 80km of their natal sites, in the northern USA 43.7% and 27.7% of juveniles were found more than 160km and 320km from their hatching locations, respectively (Stewart 1952). Moreover, there is currently no evidence for barn owls in North America showing bias in dispersal direction (Taylor 1999). These spatially random movements, in combination with their long distance dispersing behaviour, is what likely contributes to their high levels of gene flow, and hence the lack of genetic structuring.

Similar results were found in a separate population genetics study of barn owls in Europe, where the global F_{ST} value was even lower (0.011) across its distribution from Evora, Portugual to Budapest, Hungary (Antoniazza et al. 2009). Extensive introgression among haplotypes was also observed in barred owls (*Strix varia*), another long-dispersing owl species in North America known to be associated with suburban settings (Barrowclough et al. 2011). However, other highly vagile, non-migratory owl species in North America have exhibited clear population and/or phylogeographic structuring patterns across their range, including great grey owls (*Strix nebulosa*, Hull et al. 2010), spotted owls (*Strix occidentalis*, Haig et al. 2004), and western screech owls (*Megascops kennicottii*) and eastern screech owls (*Megascops asio*, Proudfoot 2007). Unlike barn and barred owls, these owl species do not adapt well to anthropogenically altered landscapes, but rather occur primarily in woodland habitats. Discontinuity in their habitats as a result of widespread deforestation ultimately limits gene flow, and hence potentially creates more pronounced population structuring in these owl species. In contrast, historic conversions of forests into farmlands may have provided enhanced dispersal corridors for barn owls, allowing for improved connectivity among populations, and thus resulting in the small F_{ST} value calculated from this study.

Genetic divergence in the Santa Barbara Island population

Microsatellite data clearly revealed genetic differentiation between Santa Barbara Island barn owls and all other sampled regions. This divergence in genotype and allele frequency demonstrated that migration rate from mainland California is restricted, suggesting that the Santa Barbara Island barn owls are functionally independent (Moritz 1994), and that its viability depends very little on immigration from its mainland counterpart. Accordingly, I recommend that the Santa Barbara Island barn owl population should be designated as a Management Unit (MU). Despite the strong evidence for divergence in allele frequency, Santa Barbara Island barn owls did not contain any distinctive haplotypes, nor were they reciprocally monophyletic. This suggests that occasional gene flow throughout its evolutionary history prevented the formation of an unique and isolated lineage. Accordingly, a status of Evolutionarily Significant Unit (ESU) cannot be assigned to them. While my study detected two individuals tagged as migrants, it is worth noting that this assignment is based on a population genetics perspective. In other words, the two owls contain immigrant ancestry from elsewhere in North America, but did not immigrate to the island per se.

Relocating Santa Barbara Island barn owls to mainland California has been suggested as a strategy to decrease predation on Scripps's murrelet (Millus et al. 2007, Nur et al. 2013, S. Thomsen pers. comm.). That may be a possible management option from a genetics perspective since (1) the population of Santa Barbara Island barn owls cannot be recognized as a ESU, and (2) barn owl emigration rates from mainland California is restricted. Given that the strait between the Channel Islands and mainland California is evidently acting as a physical barrier for dispersal, the rate of recolonization of the island from mainland would be in theory quite low. Moreover, given the island's relatively small area (2.63km^2), any individuals from mainland California "bumping into" such a small target via random dispersal would be a rare occurrence, making population re-establishment relatively difficult. This phenomenon, known as the "target area effect", is commonly established in island biogeography theory (Buckley and Knedlhans 1986). However, since migration rates of barn owls residing on the other

Channel Islands are unknown, additional genetic profiling from the archipelago would be necessary to fully understand the demography of barn owls on Santa Barbara Island.

Genetic diversity in a northwestern peripheral population

Barn owls from mainland BC, despite being situated at the edge of their distribution range and experiencing habitat degradation, do not exhibit lower genetic diversity when compared to other larger, more stable populations in the USA, including Oregon, California, and Pennsylvania. That suggests the mainland BC population is well connected with its southern neighbouring populations, allowing regular exchange of alleles to maintain genetic diversity. Furthermore, parent-offspring or full sibling matings is rare as females disperse longer distances than males from natal sites (Taylor 1994). The lack of inbreeding with close relatives decreases the chance that individuals would share identical alleles within a population, and consequently promotes the overall genetic variation.

In contrast, barn owls on Vancouver Island are genetically depauperate in comparison to other continental populations. As a result of being an island and a peripheral population, lower genetic diversity is expected (Frankham 1997, Hoffman and Blows 1994). Microsatellite results demonstrated that gene flow from the mainland is somewhat restricted as indicated by the low, but significant, F_{ST} value found between BC/WA and Vancouver Island. The patchy nature of their habitat on Vancouver Island is a typical characteristic in peripheral populations. Accordingly, the density of barn owls is lower than that of the Lower Mainland (COSEWIC 2010). As lower effective population size is often associated with restricted gene flow (Hoffman and Blows 1994), inbreeding can potentially become more prevalent. Over time, as alleles become fixed in the population, genetic diversity is also reduced. However, the islets southeast of Vancouver Island (i.e. San Juan and Gulf Islands) may moderately facilitate dispersal from the mainland, and consequently individuals established on Vancouver Island are still genetically similar to the mainland populations as a result.

While it is clear that the genetic diversity of island populations is in general lower than their mainland counterparts (Frankham 1997), this phenomenon does not hold true for all populations situated near the edge of a species distribution (Vucetich and Waite 2003; Eckert et al. 2008). When migration rate is high, substantial genetic variation is maintained in a peripheral population despite typically having higher genetic drift associated with a lower effective population size (Vucetich and Waite 2003). On the other hand, when there is restricted gene flow from core populations, as in cases where there is an existing geographic barrier, the negative effects of being a peripheral population on genetic diversity become more pronounced (Vucetich and Waite 2003). This study on the two peripheral populations of barn owls presents both sides of this phenomenon: the higher genetic diversity of the BC mainland population is attributed to its strong connectivity with the core US populations, whereas the Vancouver Island population contained fewer alleles and reduced heterozygosity, likely as a result of limited gene flow

Table 2.1 – Characterization of eight barn owl microsatellite loci from Burri et al. (2008). At least one primer (either the forward or reverse) from each primer set was tailed with a M13 Primer of 19-20 base pairs (LICOR Inc.). Listed for each locus are number of individuals genotyped (N), number of alleles (A), expected heterozygosity (H_{exp}), and size range of alleles.

Locus	N	A	H_{exp}	Size range (bp)
TA216	126	12	0.707	197-229
TA413	126	17	0.845	167-243
TA402	126	16	0.849	193-261
TA408	126	9	0.577	213-265
TA212	126	4	0.655	269-281
TA215	125	9	0.581	310-330
TA305	126	3	0.437	195-210
TA414	125	25	0.906	321-435

Table 2.2 – Pairwise comparisons of genetic differentiation between sampling regions of barn owls (*Tyto alba*) in western North America and Pennsylvania. Pairwise $\mathbf{F_{ST}}$ estimates (below diagonal) between all seven regions were calculated using eight microsatellite loci. $\mathbf{H_{ST}}$ values (above diagonal) between BC, SB Isl, South CA and Pennsylvania were calculated using NAD2 mitochondrial region sequence data (1010 base pairs). All bolded numerical values indicate statistical significance after a False Discovery Rate (FDR) correction. P-values before the FDR correction are indicated by asterisk(s).

Sampling region	BC / WA	Van Isl	West OR	East OR	SB Isl	South CA	Pennsylvania
BC / WA	--------	n/a	n/a	n/a	**0.17****	0.03	0.02
Van Isl	**0.02***	--------	n/a	n/a	n/a	n/a	n/a
West OR	0.01	0.01	--------	n/a	n/a	n/a	n/a
East OR	0.01	0.00	0.00	--------	n/a	n/a	n/a
SB Isl	**0.05*****	**0.08*****	**0.07*****	**0.06*****	--------	0.07	**0.16****
South CA	0.01*	0.01	0.00	0.01	**0.06*****	--------	-0.02
Pennsylvania	**0.02****	0.02*	0.02*	**0.03****	**0.08*****	**0.03****	--------

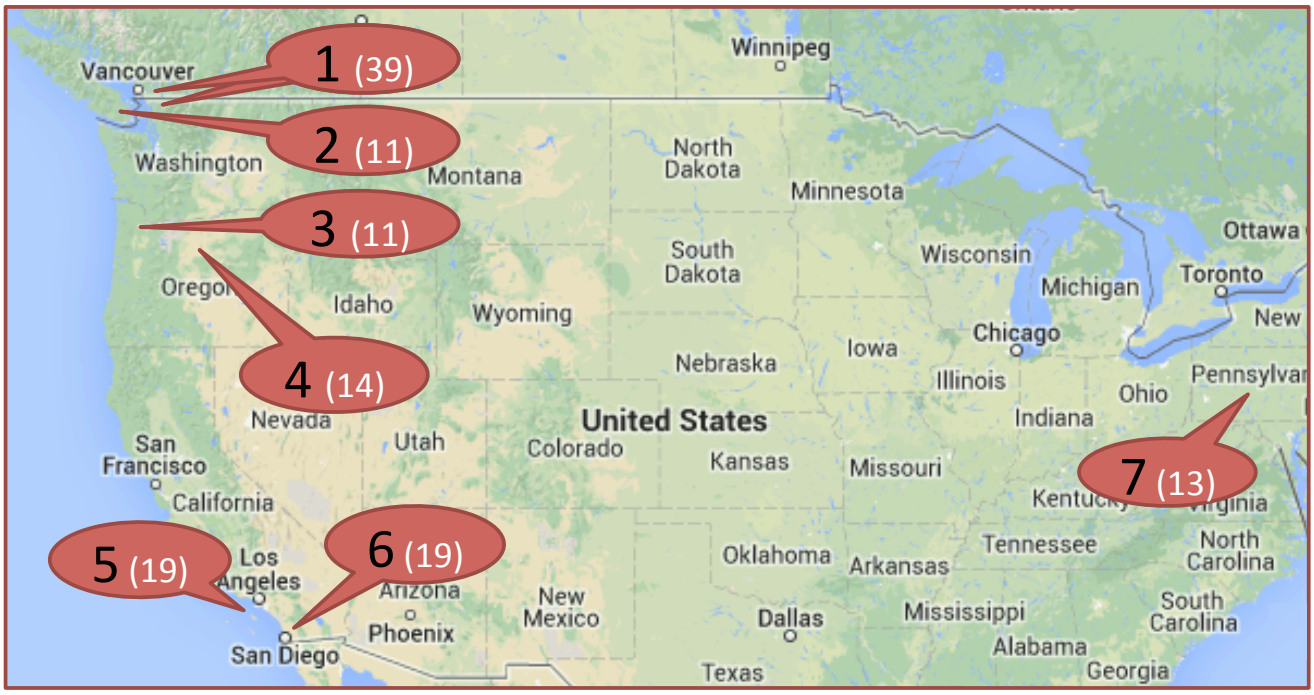
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$
n/a: data not available

Table 2.3 – Proportion of individuals (expressed in %) from the sampling regions that were assigned to corresponding inferred regions using population assignment analysis. Monte Carlo test (10000 permutations) was used to generate a null-distribution of likelihood values with which the values for the sampled individuals were compared. An individual was assigned to a certain inferred region when its likelihood value is the highest for that particular inferred region. Values across the Santa Barbara (SB) Island row are bolded to emphasize that most individuals (89.5%) were properly assigned to their sampling region.

Sampling region	Inferred region						
	BC / WA	Van Isl	West OR	East OR	SB Isl	South CA	Pennsylvania
BC / WA	41.0	5.1	15.4	12.8	7.7	12.8	5.1
Van Isl	27.3	54.5	9.1	9.1	0.0	0.0	0.0
West OR	27.3	0.0	27.3	18.2	0.0	27.3	0.0
East OR	14.3	14.3	21.4	42.9	0.0	7.1	0.0
SB Isl	**0.0**	**5.3**	**0.0**	**0.0**	**89.5**	**5.3**	**0.0**
South CA	26.3	10.5	5.3	0.0	5.3	47.4	5.3
Pennsylvania	30.8	0.0	7.7	0.0	0.0	23.1	38.5

Table 2.4 – Microsatellite polymorphism data for barn owls (*Tyto alba*) from 7 sampling regions. The number of individuals sampled for microsatellite data ($\mathbf{N_{ms}}$), allelic richness corrected for unequal sample sizes ($\mathbf{AR_C}$), observed heterozygosity ($\mathbf{H_O}$), expected heterozygosity ($\mathbf{H_E}$), number of individuals analyzed for mitochondrial data ($\mathbf{N_{mt}}$), number of haplotypes per population ($\mathbf{H_P}$), and haplotype diversity (**Hd**) are listed for each sampling region.

Sampling region	Microsatellite DNA				Mitochondrial DNA		
	N_{ms}	AR_C	H_O	H_E	N_{mt}	H_P	Hd
BC mainland	33	4.05	0.72	0.72	9	5	0.806
Van Isl	11	3.71	0.64	0.66	n/a	n/a	n/a
West OR	11	3.96	0.72	0.68	n/a	n/a	n/a
East OR	14	4.08	0.70	0.72	n/a	n/a	n/a
SB Isl	19	3.56	0.76	0.70	10	2	0.467
South CA	19	3.96	0.67	0.70	9	8	0.972
Pennsylvania	13	4.14	0.69	0.71	8	8	1.000



1. BC/Washington Mainland	**4.** Eastern Oregon	**7.** Pennsylvania
2. Vancouver Island	**5.** Santa Barbara Island	
3. Western Oregon	**6.** Southern California	**Total N = 126**

Figure 2.1 – Regions where barn owl (*Tyto alba*) whole blood, feathers, or muscle tissue samples were collected in North America. Numbers in black correspond to the regions listed; numbers in white represent the sample size.

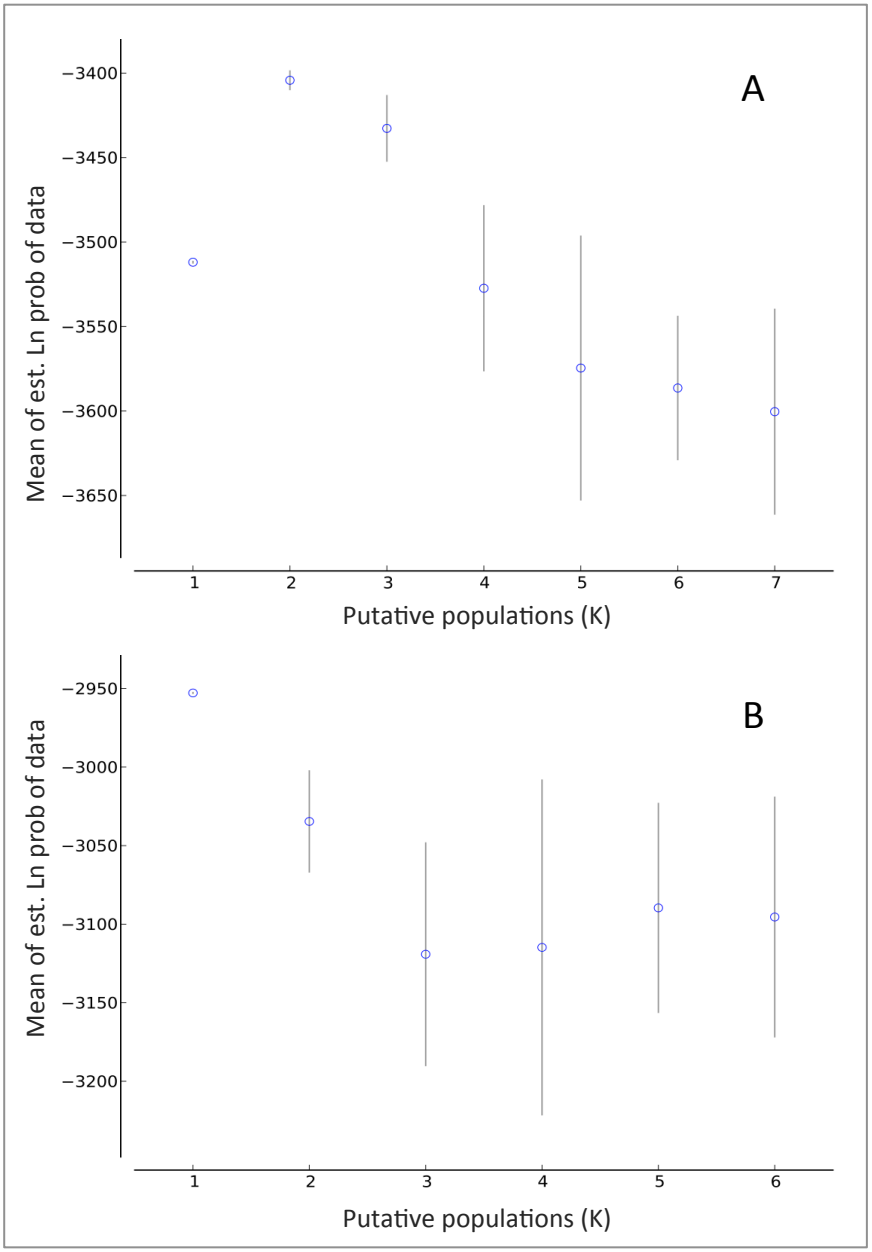


Figure 2.2 – STRUCTURE analysis**.** Posterior probability of population membership from STRUCTURE for **(A)** 1 to 7 putative populations (all 126 individuals included) and **(B)** 1 to 6 putative populations (excluding Santa Barbara Island individuals) of barn owls (*Tyto alba*) sampled in western North America and Pennsylvania. Each value is a mean of 20 STRUCTURE simulations of variation across 8 microsatellite DNA loci (error bars are standard deviations).

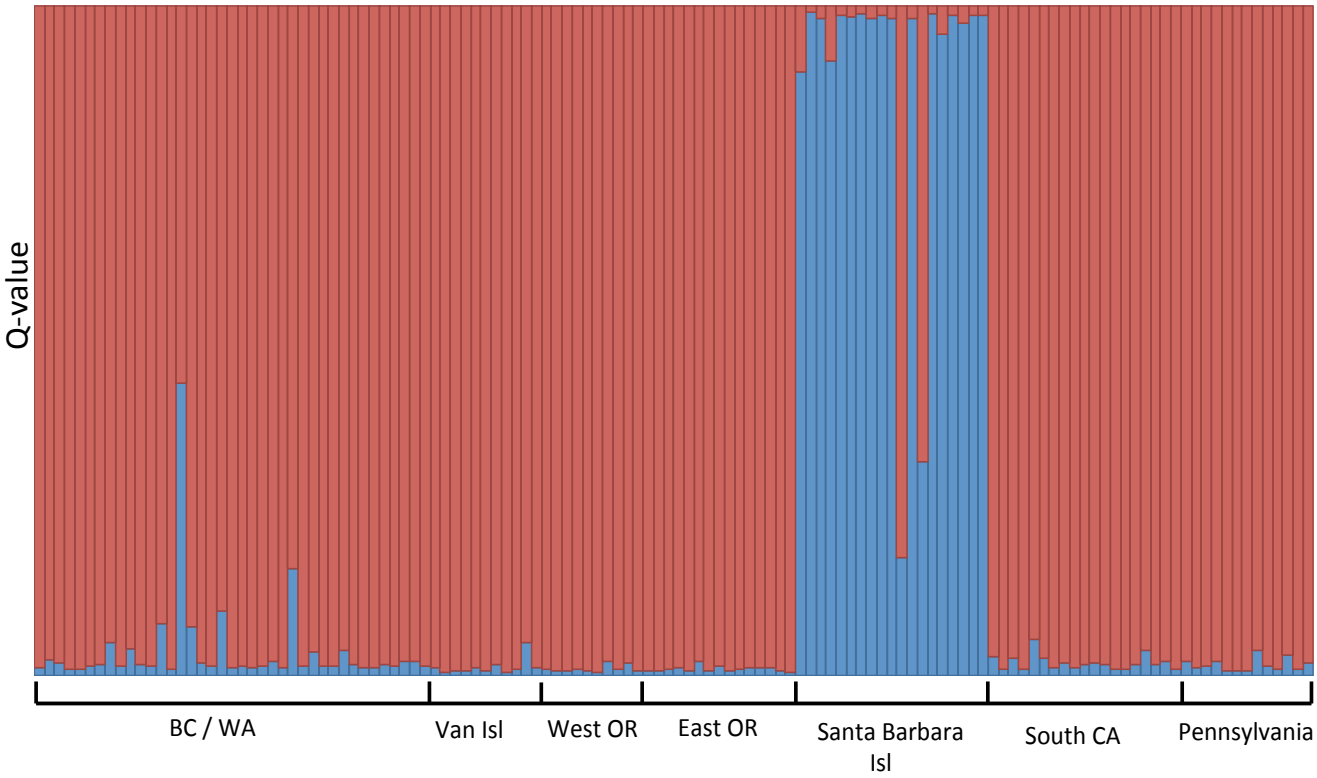


Figure 2.3 – Summary output of 20 independent runs in STRUCTURE from barn owls (K = 2, N = 126). Assayed using 8 microsatellite markers, each individual is represented by a thin vertical line, the height of which shows its admixture coefficient (Q-value). Each colour depicts a unique genetic cluster: blue represents the Santa Barbara Island lineage, whereas red represents other sampled regions. Individuals with mixed lineages are indicated by bars with both red and blue, where the proportional height of each coloured section represents the proportional contribution of the 2 lineages.

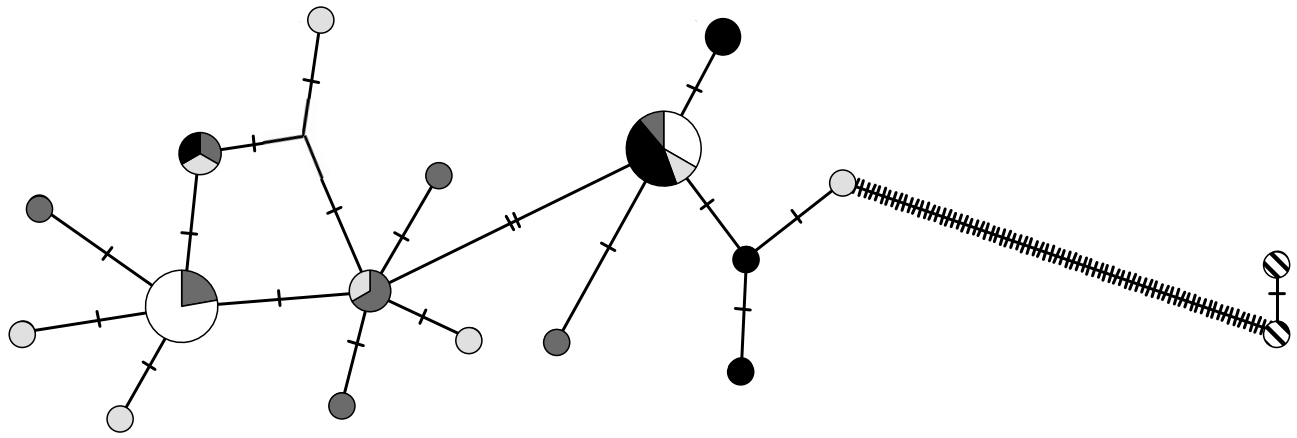


Figure 2.4 – Median-joining haplotype network for 37 North America barn owls (*Tyto alba pratincola*) showing low levels of phylogeographic congruence between the four sampled regions: *black* = British Columbia, *dark grey* = Southern California, *light grey* = Pennsylvania State, and *white* = Santa Barbara Island. Australian barn owls (*Tyto alba delicatula*) are assigned as the outgroup, as represented by dashed circles on the far right. Area of circles correspond to frequency of haplotype and slices of circles indicate relative number of individuals from each location with that haplotype. Dashes indicate a single nucleotide difference.

CHAPTER 3 – USING THE CYP2C45 GENE TO EXPLAIN DIFFERENTIAL SENSITIVITY TO RODENTICIDES IN BARN OWLS

3.1. Introduction

The removal of xenobiotic substances from an organism's body is a vital physiological process found in all vertebrates to prevent the buildup of toxic chemicals. Detoxification usually occurs in the liver, where a highly diverse superfamily of enzymes, the cytochrome P450 (CYP), is primarily involved in the metabolism of a variety of exogenous compounds (Guengerich 2008, Nebert and Russell 2002, Mueller and Miller 1948). CYP enzymes use haem iron to oxidize foreign compounds by either adding or exposing a hydroxyl group, and thereby increasing their solubility for more effective disposal by the kidneys (Meunier et al. 2004, Ioannides and Lewis 2004). Because CYP enzymes play a broad and significant role in physiological and toxicological processes, this ancient superfamily of proteins is found in all domains of life (Meunier et al. 2004, Danielson 2002). While there are currently 977 families documented within the CYP superfamily, the CYP2 family is the largest and the most diverse of the vertebrate CYPs (Nelson et al. 2009). They are responsible for the metabolism of a wide array of complex xenobiotic chemicals such as clinical drugs and environmental toxicants (Karlgren et al. 2005, Ioannides and Lewis 2004).

Warfarin is a clinical anticoagulant, often referred to as the "blood-thinner". Human patients with thrombosis – a medical condition where blood clots congeal locally inside a vessel – are often prescribed with oral warfarin to facilitate blood flow. While warfarin does eventually become metabolized in the liver and eliminated from the body, there is individual variability in the rate of metabolism of the drug (Aithal et al. 1999, Sconce et al. 2005). Hence, dosage requirement also varies according to individual physiological tolerance. The cytochrome P450, subfamily IIC, polypeptide (CYP2C9) gene is predominantly involved in warfarin detoxification in the liver (Aithal et al. 1999, Sconce et al. 2005, Higashi et al. 2002). To date, 58 gene SNPs (Single Nucleotide Polymorphisms) in the CYP2C9 gene have been identified in humans, many of which have been shown *in vitro* and *in vivo* to be associated with reduced warfarin

metabolism (CYP2C9 allele nomenclature 2014). One of the most common variant alleles found in Caucasian populations is a substitution of cysteine (Cys) for Arginine (Arg) at amino acid residue 144 due to a transversion change from a cytosine to thymine (C>T) at nucleotide 430 (Aithal et al. 1999, King et al. 2004). Patients with this $Arg^{144}Cys$ variant allele encodes for a dysfunctional CYP2C9 enzyme, whereby the enzymatic activity decreases by 30-50%. As a result, they require a lower warfarin dose and are at a greater risk of internal bleeding (Aithal et al. 1999, Higashi et al. 2002, King et al. 2004). Meanwhile, other variant alleles found in other racial groups, such as the $L^{90}P$ and $T^{130}R$, have been shown to have an 89% and 90% reduction in enzyme activity, respectively (Guo et al. 2005, Maekawa et al. 2006).

While warfarin has been proven useful in clinical settings for treating human patients, it is also widely used as an anticoagulant rodenticide (AR) compound. Warfarin, among other first-generation anticoagulant rodenticides (FGARs), was decreasing in use due to the development of genetic resistance in rodent populations. Second generation anticoagulant rodenticides (SGARs) became more commonly employed in urban and rural settings to control rodent populations because of their highly effective single dose killing. However, new regulatory restrictions in North America on domestic use of SGARs may reverse that trend (Elliott et al. 2014). For instance, as of December 2012 SGAR products (brodifacoum and difethialone) are no longer available in the domestic retail market in Canada, and can only be used by licensed applicators (Elliot et al. 2014).

Over-dosage of these anticoagulant compounds causes severe internal hemorrhage by disrupting the vitamin K cycle, an essential biochemical process required for blood coagulation (Pelz and John 2011). Like many birds of prey that hunt extensively for small rodents, barn owls are at an increased risk of secondary poisoning from the now widespread use of SGARs in urban and rural farmlands (Albert et al. 2010, Walker et al. 2013). In fact, in a warfarin metabolism assay, owls showed the lowest ability to detoxify warfarin when compared to other avian species, which is indicative that they are a high-risk group to poisoning from AR (Watanabe et al. 2010). Although 0.1 – 0.2 mg/kg of SGAR residues in liver has been suggested to be a “potentially lethal range” (Newton et al. 1999, Newton et al. 1998), tolerance

to AR appears to be highly variable among individuals for any given avian species (Albert et al. 2010, Rattner et al. 2014). Walker et al. (2013) demonstrated that some barn owls with 0.1 – 0.337 mg/kg of SGAR residue detected in their livers exhibited no evident signs of hemorrhaging, whereas others with < 0.1 mg/kg were diagnosed as suffering from acute hemorrhaging as the cause of death. Similarly, eastern screech owls experimentally fed with diphacinone showed high individual variability in their detoxification capacity (Rattner et al. 2014). Currently, no studies have investigated the genetic basis for the apparent individual variation in rodenticide sensitivity in avian species.

The cytochrome P450 2C45 (CYP2C45) gene was the first member of the CYP2C subfamily to be cloned in avian species and has been well characterized in chickens (*Gallus gallus*, Baader et al. 2002). This gene shares considerable protein and DNA sequence identity with other CYP2Cs in non-avian animals, and hence has been clustered in the CYP2C subfamily (Baader et al. 2002). The National Center for Biotechnology Information (NCBI) RNA reference sequences collection (RefSeq) has designated the gene as a homolog to the human CYP2C9 gene (Pruitt et al. 2002).

Understanding of the function of the CYP2C45 gene in avian species is limited. Using real-time PCR, Watanabe et al. (2013) has shown that when compared to nine other major avian CYP isoforms, CYP2C45 exhibited the highest basal mRNA expression in chicken liver, suggesting that CYP2C45 may be the dominant isoform in avian xenobiotic metabolism. *In vitro* experiments demonstrated that the CYP2C45 gene in chickens is highly induced by phenobarbital, a nervous system and brain-related pharmaceutical drug used as a sedative (Baader et al. 2002). In comparison, another study showed a negative correlation between the expression of this gene in great cormorants (*Phalacrocorax carbo*) and higher environmental concentrations of perfluorooctane sulfonate, a pollutant used extensively in industrial and household applications (Kubota et al. 2010). There is, however, currently no knowledge of the gene's associations with warfarin metabolism in birds. In Chapter 3, I used the CYP2C45 gene to investigate whether single point mutations in this gene that could be associated with differential susceptibility to rodenticide in barn owls. Specifically, I aimed to (1) determine the

locations of single nucleotide polymorphisms (SNPs) in the CYP2C45 gene of barn owls, and (2) assess whether these nucleotide changes could be associated with reduced or increased susceptibility to anticoagulant rodenticides (AR).

3.2. Methods

I acquired muscle tissue samples from a subset of 20 barn owl carcasses collected from 2012-2013 from the Lower Mainland, British Columbia, each with existing necropsy and rodenticide residue data (Appendix A). Rodenticide residue analysis from liver samples was conducted at the Environment Canada National Wildlife Research Centre in Ottawa, Ontario, Canada. FGAR compounds tested included diphacinone, chlorophacinone, pindone, and warfarin; SGAR compounds included brodifacoum, bromadiolone, and difetialone. Post-mortem evaluations were conducted by an experienced wildlife veterinary pathologist. Any pathophysiological symptoms of toxicosis, such as hemorrhage or anemia in the absence of traumatic injury or infectious/parasitic diseases, were documented.

Based on a liver residue concentration for total AR of 0.1 mg/kg to be indicative of lethality for barn owls (Newton et al. 1998), I used this value as a threshold to categorize individuals into "high/low AR tolerance" . I defined "high AR tolerance" as barn owls with total AR residue of > 0.1mg/kg while showing no toxicosis symptoms. In contrast, those with total AR residue of < 0.1mg/kg with presence of toxicosis symptoms would be designated as "low AR tolerance".

From each of the 20 samples, I extracted total DNA from 5mg of muscle tissue using a modified protocol of Meulenbelt et al. (1995). DNA sequences from the CYP2C45 gene of chicken (*Gallus gallus*), wild turkey (*Meleagris gallopavo*), zebra finch (*Taeniopygia guttata*) were obtained from genome browsers: NCBI and Ensembl. I aligned the exon regions from the three avian species and identified conserved regions of approximately 20-30 base pairs. Based on these conserved sequences, I designed 6 sets of primer (Table 3.1) and amplified 6 segments of the CYP2C45 gene from the extracted barn owl DNA (Figure 3.1). PCRs were performed in a final volume of 20μL containing 10-25ng of template DNA, 1xPCR buffer, 0.2μM

of dNTPs, 0.3 U of *Taq*, and 0.1μM of each forward and reverse primer. PCR products were sequenced at the NAPS Unit, University of British Columbia, using Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Ontario, Canada) and were resolved on Applied Biosystems 3730S 48-capillary DNA analyzer. I compared barn owl DNA sequences and the translated protein sequences with chicken, zebra finch, and wild turkey to confirm that the correct ortholog gene segment was amplified. I conducted all sequence alignment, editing of chromatograms, and identification of SNPs using SeqMan Pro 8.1.

3.3. Results

Sequence comparison between barn owls and other Aves

Exons 2, 3, 4, 5 and 6 of the CYP2C45 gene were fully sequenced from all 13 barn owls. Primers were designed 30 base pairs upstream from the start codon, and hence 30 base pairs from the 5' end of exon 1 were not sequenced. Similarly, the primers that I designed sequenced a part of exon 7 (104 base pairs missing). Exons 8 and 9 were not sequenced successfully.

Amino acid sequence alignment between barn owl, chicken, finch, and cormorant showed high sequence similarity (Figure 3.2). Long stretches of identical regions (i.e. more than 10 continuous amino acids) are found in exon 2, 3, 4, 6 and 7. I also identified 6 amino acid changes that are unique to barn owls, and 2 regions where all four species display distinct amino acid variants (Figure 3.2). There is a high degree of similarity for amino acids and DNA sequences between barn owl and other avian species, with great cormorant being the highest, followed by zebra finch, and chicken (Table 3.2). No stop codons were detected in the translated barn owl amino acid sequence, suggesting that the amplified sequence is a not a pseudogene but rather a functional gene.

Single nucleotide mutations in association with AR sensitivity

Of the 20 barn owls with AR residue results, 6 had total AR concentrations below LOQ (Limit of Quantitation), and 1 with poor sequencing data due to poor quality DNA. Consequently, I used the remaining 13 barn owls in my analysis. Of the 13 individuals, 5 belong in the "low AR tolerance" category, and 3 in the "high AR tolerance" category (Table 3.3). AR concentrations were found to be highly variable for all 4 AR chemicals as well as their total (Figure 3.3). There is also significant amount of overlap between owls with and without toxicosis symptoms.

Sequencing of the CYP2C45 gene in barn owls revealed that individual "#L12-558" has a heterozygous nucleotide change at position 1011 of the transcriptome, located in exon 7 (Figure 3.4). Wild-type individuals are homozygous (C / C) at this position, whereas this particular mutant individual is heterozygous (C / T). I conducted two separate PCRs and sequenced exon 7 two additional times to confirm this heterozygous mutation. This C1011T transversion leads to an Ala344Val substitution in the encoded protein, and is located in one of the conserved regions (Figure 3.2). Necropsy results indicate that individual #L12-558 exhibited signs of secondary poisoning (hemorrhage in stomach and intestines), while toxicology analysis showed that only 0.011 mg/kg of SGAR residue was found in its liver. Accordingly, it was placed in the "low AR tolerance" category (Table 3.3).

I also identified 9 individual SNP sites among the 13 genotyped barn owls in the non-coding regions (introns). These SNPs have no apparent correlation with increased or decreased tolerance to AR (see Appendix B).

3.4. Discussion

This is the first study to have discovered a nucleotide change in the coding region of the CYP2C45 gene in a barn owl individual that appears to exhibit a low level of tolerance to AR. It involves a C-to-T transversion at nucleotide position 1011 in exon 7, causing a mutation at codon 344, which resulted in an alanine to valine substitution. Sequence comparison among

bird species revealed a highly conserved region in exon 7 (Figure 3.2), which suggests that this continuous stretch of 12 amino acids plays a critical role in the biological function and structure of the protein, such as ligand binding or protein-protein interactions. The amino acid substitution found in this particular barn owl is located in the conserved region of exon 7. As a consequence, it may have significant impacts on the protein folding pathway and its overall property.

Protein functionality can also be substantially affected when the novel amino acid has vastly different characteristics than the replaced one. Amino acid substitutions identified at critical locations in the human CYP2C9 protein are known to lead to a decrease or even an absence in enzymatic activity. For instance, as a consequence of an Asn204His substitution due to a missense mutation in exon 4, the enzyme exhibited a reduced binding affinity for coumarin drugs, and hence impeding its metabolic clearance (Nahar et al. 2013). Similarly, an Arg125Leu substitution has inhibited the interaction between the CYP2C9 protein and the CYP oxidoreductase (POR), an enzyme responsible for electron transfer from NADPH to CYP proteins (Lee et al. 2014). These two deleterious substitutions are attributed to the replaced amino acids having considerably different properties (e.g. polarity, charge) than the original ones. In comparison, however, the Ala34Val substitution in the barn owl CYP2C45 protein may have less of an adverse effect on the protein since alanine and valine share similar properties in that they both carry small hydrophobic side groups (Henikoff and Henikoff 1992, Majewski and Ott 2003). To further investigate for evidence of protein alteration, the complete gene must first be sequenced, followed by use of protein folding programs or protein crystallography to visualize its three dimensional structure.

Although a heterozygous mutation was identified in one barn owl individual with “low AR tolerance”, the same mutation was not found in the other four cases in the same category, nor were other mutations found in the “high AR tolerance” individuals. Other genetic factors can also determine AR sensitivity level in vertebrates. The vitamin K epoxide reductase complex (VKORC1) is a key enzyme in the recycling pathway of vitamin K, an essential component for the formation of blood clots (Pelz and John 2011). Previous studies have shown that variation

in warfarin dosing is due partially to polymorphisms in the gene encoding for VKORC1 (Li et al. 2006). Six unique SNPs in the VKORC1 gene were found in patients who required adjustments to their warfarin dosage to achieve a normal prothrombin time (Li et al. 2006). However, unlike the CYP2C45 gene, the VKORC1 gene has not been well characterized in avian species (e.g. gene expression profiling, protein functional analysis); in fact, the gene has yet to be fully sequenced in the assembled chicken genome (Warren et al. 2005). As a result, although I attempted to sequence the VKORC1 gene in barn owls, there was very little success mainly because I was unable to design primers specific enough to amplify the gene.

Alignment of the barn owl CYP2C45 gene with other avian species revealed substantial similarity in both nucleotide and amino acid sequences, with 5 conserved amino acid regions found between them, and only 2 positions where all four species contain a discrete amino acid. That result increases the likelihood that my novel primers successfully amplified the paralog gene in the barn owls. Barn owl DNA sequence similarity was the highest when compared to great cormorants and zebra finch (93 and 91%, respectively), and lowest when compared to chicken (86%). Chickens, among other Galloanserae species (gamefowl and waterfowl), formed a basal clade to the Neoaves (all other birds except for ratites and tinamous), which may explain the relatively lower sequence identity between barn owl and chicken. In contrast, 6 amino acid changes were identified to be unique to barn owls, which is suggestive that these positions could attribute to any potential interspecific differences in the enzymatic activity between the barn owl CYP2C45 protein and other avian species.

It is worth noting that categorization of AR tolerance based on the 0.1 mg/kg threshold value (Newton et al. 1998) has a degree of uncertainty. Thomas et al. (2011) suggested that rather than using that defined lethal threshold, it is more appropriate to estimate the probability of toxicosis based on AR liver residue. Using a logistic regression approach, they estimated that approximately 10-20% of barn owls are likely to suffer mortality within the 0.1 – 0.2 mg/kg range. Another concern is that depending on the type of chemical and the concentration ingested, animals typically stay alive for several days after a lethal dose of AR (Meehan 1984). Also, we do not know what proportion of AR residues could have been cleared from the liver

before dying. Eastern screech owls (*Megascops asio*), for instance, initiate rapid clearance of the FGAR, diphacinone, by day 2 of post-exposure, with a half-life of 0.88 days (Rattner et al. 2014). There is limited information on the pharmacokinetics of SGARs in birds. Furthermore, owls classified as “high AR tolerance” may have simply ingested a high dose of AR, but died as a result of non-AR related causes. Thus, classifying individuals as “low” or “high” AR tolerance is fraught with uncertainties due to these variables.

While this study has detected a mutation in the CYP2C45 gene in one barn owl individual who exhibited low AR tolerance, the biological relationship between this gene and AR metabolism has yet to be established experimentally. Watanabe et al. (2010) proposed that the CYP2C45 enzyme is the primary CYP protein responsible for the clearing of xenobiotic compounds. Saengtienchai et al. (2011) on the other hand suggested that aldehyde oxidase is the predominant enzyme in chickens that biotransforms drugs and xenobiotics, including warfarin and other coumarin derivatives. Clearly, the detoxification process of coumarin compounds in birds is still shrouded in uncertainty. A mechanistic understanding is needed to explain the apparent inter- and intraspecific variation in sensitivity to AR. Further investigations are needed to confirm whether this protein is indeed responsible for the metabolism of coumarin compounds in birds. Additional studies can include coumarin metabolism assays, measurement of mRNA expression levels upon AR exposure, protein crystallography, and controlled experimental studies using model avian organisms such as chickens. Further characterizing and determining the precise functional mechanisms of the CYP2C45 gene, from gene expression to protein activity to the biochemical pathway, would allow us to gain new insights into the metabolism of environmental toxicants in avian species.

Table 3.1 – Forward and reverse primers used for amplification of CYP2C45 segments from barn owls (*Tyto alba*). Also included are expected amplicon sizes, and PCR annealing temperatures. The A-F segments correspond to Figure 3.1.

Segment	Primer sequences (5' - 3')	Amplicon sizes (bp)	Annealing temperatures (°C)
A	F: CTCCTGGTTTGCATTGCTTGCCT R: TGTCCTCTGGCAGCAAACTC	633	55
B	F: GGTGAAAGAAGCCTTGGTCGATC R: CAATGCTCCTCTTCCCCATCCCAAA	635	53.5
C	F: GCATTATTTTCAGCAACAACGAGGG R: TGTTGTTCATGTTGTTCATCAG	517	55
D	F: GACTATAAAGACAAGAAGTTCC R: GGAAGCAGTCAATGAAATCCTG	664	60
E	F: CAGGATTTCATTGACTGCTTCC R: GTGCTTGTTGTCTCCGTTCCAGC	659	61.5
F	F: GCTGGAACGGAGACAACAAGCAC R: GTGCTTGTTGTCTCCGTTCCAGC	589	61.5

Table 3.2 – CYP2C45 DNA and amino acid sequence percent similarity compared between barn owls (*Tyto alba*) and three other avian species: chicken (*Gallus gallus*), zebra finch (*Taeniopygia guttata*), and great cormorant (*Phalacrocorax carbo*). Values are expressed in percentages.

	Chicken	**Zebra finch**	**Great cormorant**
DNA	85.7	91.1	93.4
Amino acid	83.1	89.8	92.7

Table 3.3 – Concentration (mg/kg) of anticoagulant rodenticide (AR) residues in barn owl livers and the presence/absence of toxicosis symptoms. Diphacinone (DP) was the only detectable first generation AR. Total AR concentration was calculated by summing all diphacinone and second generation AR compounds, including brodifacoum (BF) bromadiolone (BD), and difetialone (DF). Toxicosis symptoms are present when the individual was diagnosed with gastric and/or intestinal hemorrhage. Individuals highlighted in red are designated as "low AR tolerance", whereas in blue are "high AR tolerance", based on the liver residue concentration of 0.100 mg/kg as suggested by Newton et al. (1998). Concentrations too low to be detected are indicated by <LOQ (Limit of Quantitation).

Individual ID	DP	BF	BD	DF	Total AR	Toxicosis Symptoms
L12-558	<LOQ	0.011	<LOQ	<LOQ	0.011	Present
L12-559	< LOQ	< LOQ	< LOQ	0.093	0.093	Present
L12-709	<LOQ	<LOQ	<LOQ	0.081	0.081	Present
L12-736	<LOQ	<LOQ	<LOQ	0.022	0.022	Present
L12-753	<LOQ	<LOQ	0.003	<LOQ	0.003	Present
L12-727	0.021	0.003	0.043	0.170	0.237	Absent
L12-737	<LOQ	0.079	0.002	0.070	0.151	Absent
L12-738	<LOQ	0.004	0.067	0.101	0.171	Absent
L12-555	<LOQ	0.008	0.017	0.016	0.041	Absent
L12-732	<LOQ	<LOQ	<LOQ	0.042	0.042	Absent
L12-733	<LOQ	0.020	0.013	<LOQ	0.034	Absent
L12-752	<LOQ	<LOQ	0.011	0.022	0.032	Absent
L12-949	<LOQ	0.052	0.067	0.092	0.211	Present*

*It is unclear whether signs of gastric and intestinal hemorrhage in this individual are directly associated with toxicosis or not as it was also diagnosed with fractured and dislocated bones (i.e. physical trauma).

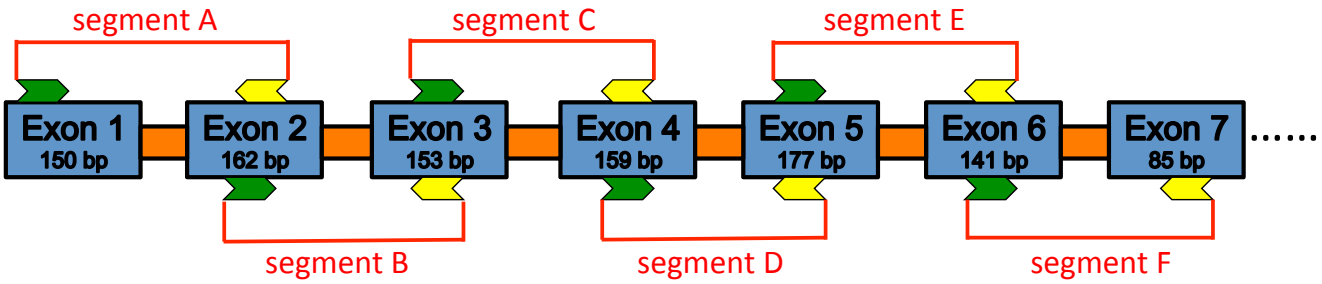


Figure 3.1 – Diagram of the barn owl (*Tyto alba*) CYP2C45 gene. The blue blocks indicate exon regions and their corresponding sizes; orange blocks indicate the introns; green and yellow arrowheads are the forward and reverse primers, respectively, used to amplify the six segments (see Table 3.1). Drawing is not to scale of actual sequence sizes. The remaining exons (8 and 9) and introns were not sequenced.

Exon	Species	Amino acid sequence	
1	TA	----------LLVCIACLLSFAAWKGRSGKGKMPPGPAPLPILGNVLQVKPKNLAKTFQK	60
	GG	MLLLGAASVVLLVCVACLLSIVQWRKRTGKGKMPEGPTPLPIVGNILEVKPKNLAKTLEK	
	TG	MELLGGVTVVLLVCIACLLSFAAWKGRSGKGKMPPGPAPLPILGNLLQVKPSNMTKTLQK	
	PC	MELLGAGTVVLLVCIACLLSVAAWRRRSGKGKMPPGPAPLPILGNVLQVKPKHLAKTLQK	
2	TA	LSEEYGPVFTVHLGSDPVVVLHGHDVVKEALVERADEFAARGHMPIGDRANNGL	114
	GG	LAEKYGPVFSVQLGSTPVVVLSGYEAVKEALIDRADEFAARGHMPIGDRANKGL	
	TG	LSEEYGPVFTVHLGSDPVVVLYGHDVVKEALVDRADEFAARGHMPIGDRTNKGL	
	PC	LSEEYGPVFTVHLGSDPVVVLHGHDVVKEALVDRADEFAARGHMPIGDRANNGL	
3	TA	GIIFSNNEEWLQVRRFALSTLRSFGMGKRSIEERIQEESDYLLEEINKTKG	165
	GG	GIIFSNNEGWLHVRRFALSTLRNFGMGKRSIEERIQEEAEHLLEEITKTKR	
	TG	GIIFSNNELWLQGRRFSLTTLRNFGMGKRSIEERIQEESDYLLEEINKTKS	
	PC	GIIFSNNKEWLEVRRFALSTLRNFGMGKRSIEERIQEETEYLLEEINKTKG	
4	TA	TPFDPTFILSCAISNVVCSIVFGKRYDYKDKKFLALMNNMNNIFEMVNSHWGQ	218
	GG	LPFDPTFKLSCAVSNVICSIVFGKRYDYKDKKFLSLMNNMNNTFEMMNSRWGQ	
	TG	TPFDPTFMLSCAVSNVICSIVFGKRYDYKDKKFLALMNNMNNIFEMMNSRWGQ	
	PC	TPFDPTFTLGCAVSNVICSIVFGKRYDYKDKKFLALMTNMNNIFEMMNSHWGQ	
5	TA	LYQMFSNILDYLPGPHNKIFTEFDALKAFVSEEVKMHQDSLDPSSPQDFIDCFLSKMQE	277
	GG	LYQMFSYVLDYLPGPHNNIFKEIDAVKAFVAEEVKLHQASLDPSAPQDFIDCFLSKMQE	
	TG	LYQMFSNILDYLPGPHNNIFAEFDALKAFVAEEVKLHQASLDPSSPQDFIDCFLCKMQE	
	PC	LYQMFSRILDYLPGPHNKIFDEFDALKAFVSEEVKIHQASLDPSSPQDFIDCFLSKMQE	
6	TA	EKEHPNSSFHMKNLITSTFDLFIAGTETTSTTIRYGLLLLLKYPKIQ	324
	GG	EKDNPKSHFHMTNLITSTFDLFIAGTETTSTTTRYGLLLLLKYPKIQ	
	TG	EKDRPNSSFYMKNLITSTFDLFLAGTETTSTTLRYGLLLLLKYPKIQ	
	PC	EKEHPNSSFHMKNLITSTFDLFIAGTESTSTTIRYGFLLLLKYPKIQ	
7	TA	EKVQEEIDQVVGRSRRPCVADRTQMPYT.....	352
	GG	EKVQEEIDRVVGRSRRPCVADRTQMPYT.....	
	TG	EKIQEEIDQVVGQSRKPCVADRTQMPYT.....	
	PC	EKVQEEIDWVVGRSRRPCVADRTQMPYT.....	

Figure 3.2 – Alignment of amino acid sequences of the CYP2C45 between barn owl (**TA**, *Tyto alba*), chicken (**GG**, *Gallus gallus*), zebra finch (**TG**, *Taeniopygia guttata*) and great cormorant (**PC**, *Phalacrocorax carbo*). The numbers on the right indicate the amino acid position at the end of each exon. Regions that are highlighted in red are long stretches (>10) of conserved amino acid sequences; in yellow are amino acid changes unique to barn owls; in green indicates that all 4 species have unique amino acids at that particular position. Chicken sequence was retrieved from Baader et al. (2002). Zebra finch and great cormorant sequences were retrieved from the Ensembl Genome Browser.

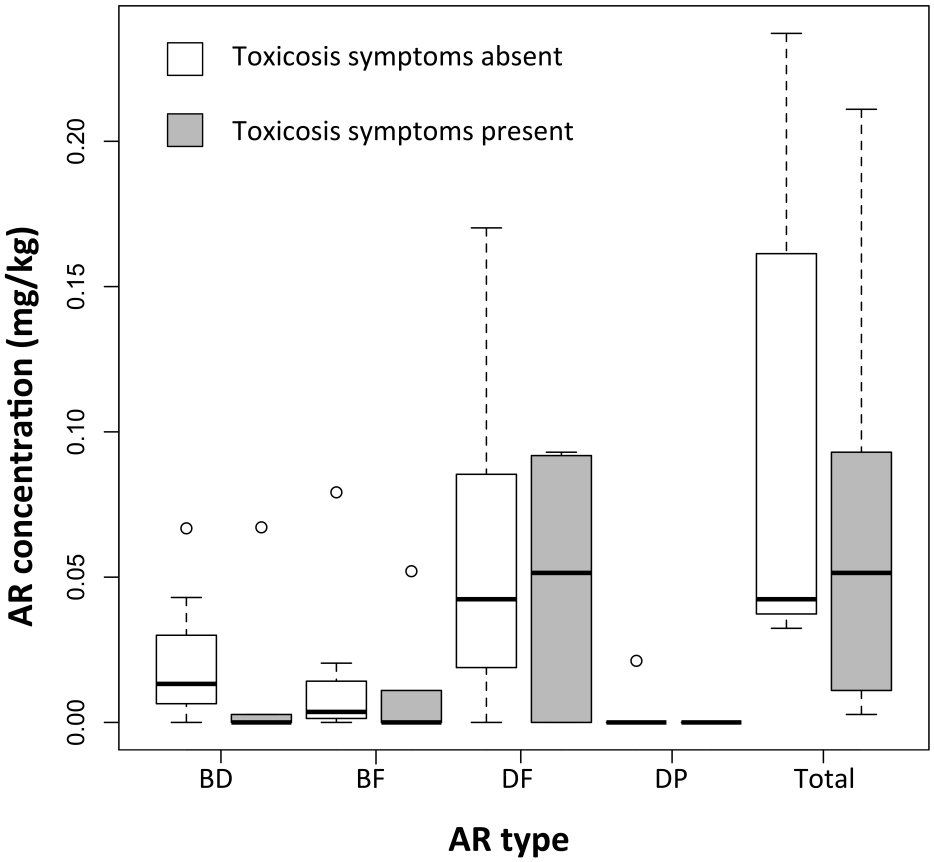


Figure 3.3 – Boxplots showing the liver residue concentrations (mg/kg) of 4 types of anticoagulant rodenticide (AR) and their total concentration detected from 13 barn owls: bromadiolone (BD), brodifacoum (BF), difethialone (DF), and diphacionone (DP). Shaded in grey are AR symptomatic individuals (N=7), whereas white are individuals with no apparent toxicosis symptoms (N=6).

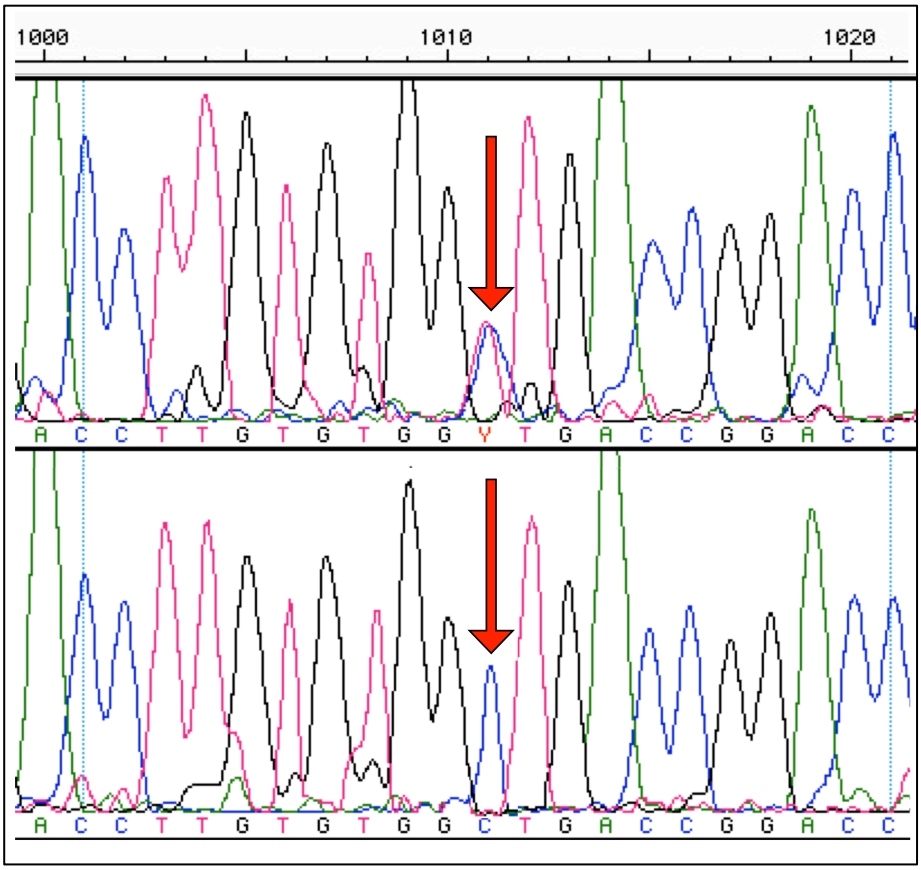


Figure 3.4 – DNA sequence chromatogram showing a single-point mutant allele identified in exon 7 of the CYP2C45 gene in barn owls (*Tyto alba*). The mutant individual "L12-558" (upper) is heterozygous at nucleotide position 1011 of the transcriptome (C / T or "Y"), whereas the wild type (lower) is a homozygous at the same nucleotide position (C / C), as indicated by the red arrow.

CHAPTER 4 – GENERAL CONCLUSIONS AND CONSERVATION IMPLICATIONS

4.1. Conservation implications for North American barn owls

In Chapter 2, using microsatellite markers and mitochondrial sequence data I demonstrated that there is in general a high degree of gene flow between barn owl populations in continental North America. This is in part owing to the connectivity of their grassland and agricultural habitat, allowing relatively unrestricted migration between populations. In addition, as highly vagile owl species, their ability to disperse long distances (on average 103 km, Marti 1999) has also likely contributed to the apparent lack of population structure. These factors have as a result contributed to the maintenance of high genetic diversity observed in the BC mainland population despite its peripheral location and ongoing habitat degradation. In this context, the mainland BC population of barn owls is well connected with its neighbouring southern populations from a population genetics perspective. Given their strong dispersal ability but recent significant declines, it is vital to focus on conserving the remaining old-field and grassland habitats in the Lower Mainland of British Columbia to allow successful immigration from nearby US populations. The Vancouver Island population, however, has less suitable habitat and a resulting lower density of barn owls. With the already limited gene flow from core populations, the preservation of suitable nesting and foraging habitat for barn owls on Vancouver Island and the nearby islets is even more crucial to prevent further inbreeding.

A previous radio-telemetry study showed that barn owls on Santa Babara Island disperse locally, and have not been recorded to fly off the island (Thomsen et al. 2014). My microsatellite analysis, similarly, demonstrated that gene flow between the Santa Barbara Island population and its mainland counterpart is substantially restricted. The unique genetic cluster detected in this study indicates that the insular population is functionally independent, and that migrants from mainland contribute minimally to the overall genetic make-up of the population. These results have crucial management implications for the barn owls. Several studies and seabird conservation groups have made proposals to relocate the Santa Barbara Island barn owls to mainland California in order to help the persistence of one of their prey

items, Scripps's murrelets (Millus et al. 2007, Nur et al. 2013, S. Thomsen pers. comm.). Given that the strait between the Channel Islands and mainland California appears to be acting as a natural barrier for dispersal, the rate of recolonization of the island would be quite low. While barn owls are able to disperse long distances from their natal site, in a study where aviary-bred barn owls were released in uninhabited nest boxes, the furthest dispersal distance was only 30 km away from the release site (Meek et al. 2003). In this context, the best option may be to translocate Santa Barbara barn owls to unoccupied territories in the mainland, which would then further reduce the possibility of them returning to the island. However, I strongly recommend genotyping barn owls from other Channel Islands in order to more fully understand the dispersal pattern within the archipelago, and thereby allowing for more informative management decisions to be made.

4.2. The CYP2C45 gene and future directions

The cytochrome P450 (CYP) superfamily of genes are known as one of the major enzyme complex involved in the metabolism of xenobiotic compounds, such as natural toxins, environmental toxicants and clinical drugs (Guengerich 2008). SNPs in the CYP2C9 gene have been identified as the factor determining warfarin sensitivity in humans. The CYP2C45 gene, like the CYP2C9 gene, has been grouped in the CYP2C subfamily (Baader et al. 2002), and is found exclusively in avian species. In Chapter 3, I identified a single point mutation in the CYP2C45 gene of an individual barn owl with apparent higher susceptibility to AR. The heterozygous change involving a C to T transversion resulted in an amino acid substitution from alanine to valine in a highly conserved region of exon 7. This suggests that there are potentially downstream consequential and adverse effects on the activity and structure of the protein. However, it is also possible that this amino acid substitution may not significantly change the protein's folding properties since alanine and valine both share non-polar characteristics.

While the CYP2C45 gene has been shown to have the highest basal mRNA expression level compared to other cytochrome P450 genes in avian species (Watanabe et al. 2013), currently

the precise mechanistic role that this gene plays in AR metabolism is unknown. Therefore, my study does not provide any direct evidence that the apparent low AR tolerance is attributed to the single point mutation found in the CYP2C45 gene. In general, there is an overall lack of understanding in the role of the CYP2C45 gene. Many future studies can be undertaken to elucidate its relationship with AR. I suggest that the next logical step would be to use a model avian organism, such as *Gallus gallus* or *Taeniopygia guttata*, to measure the liver mRNA expression level of the CYP2C45 gene upon exposure to AR compounds or other coumarin derivatives. If individuals exposed to AR compounds express elevated mRNA levels, then this would corroborate the association between the CYP2C45 gene and AR metabolism in avian species. Alternatively, given the complexity of any biochemical pathway, targeting several genes and measuring their gene expressions with correlation to various levels of susceptibility could be another possible way to explore this subject. Clearly, there is a need to further investigate the molecular basis behind variation in AR metabolism in avian species, ranging from gene regulation, mRNA expression, to cellular pathways. By continuing to shed more light on these molecular mechanisms, it would ultimately allow biologists to identify which avian species are at a higher risk to AR poisoning based on their species-specific physiological processes.

4.3. Genetics in conservation and wildlife management

The application of genetics has proven to be extremely valuable for resolving many fundamental questions in wildlife conservation and management. Advances and developments in genetic technologies have provided the opportunity to understand how environmental features or anthropogenic disturbances structure genetic variation at the population level. For instance, using non-invasive sampling of hair and genetic methods, wildlife managers were able to evaluate whether artificial corridors facilitated gene flow for bears (Frosch et al. 2014, Dixon et al. 2006). At a grander scale, quantitative phylogenetic approaches were used to identify “Evolutionary Distinct and Globally Endangered” (EDGE) avian species, allowing conservationists worldwide to recognize regions of particular value for safeguarding evolutionary diversity (Jetz et al. 2014).

While the synergy between genetics and evolutionary ecology is widely recognized, genetic tools are also immensely useful in wildlife forensics. Often when tissue samples opportunistically collected in the field are unrecognizable, DNA fingerprinting methods can be used to determine the species, or even the population, that they belong to. This approach has great potential in identifying bird species involved in aircraft collisions (Dove et al. 2010) or wildlife species vulnerable to poaching or illegal trade (Manel et al. 2002). It enables wildlife managers to determine which species or populations are at risk of experiencing anthropogenic pressures, and ultimately allocate additional conservation efforts to them. Advancements in genetic technology are also continuously being incorporated into genetic ecotoxicology. "ToxChip PCR Arrays", a DNA microarray where the expression levels of large numbers of genes are simultaneously measured, are currently under development to tailor to "sentinel species" for assessing and monitoring environmental contaminants (Porter et al. 2014). This rapid screening method would allow quantification and identification of genes that become upregulated or downregulated in response to various contaminant stressors.

Currently, however, the application of genetics has not been widely incorporated in decision-making for wildlife management and conservation. Sarre and Georges (2009) argue that the cost and time-consuming nature of genetics laboratory work is perhaps the foremost reason why wildlife managers are hesitant to take this approach. For example, optimization of PCR reactions for widely used markers such as microsatellite is time consuming, and without any expert assistance, a substantial amount of money could be spent. Additionally, wildlife management is largely field-based and focused on behavioural and ecological studies, whereas genetic studies for the most part operate in a laboratory setting. The absence of overlap between the two disciplines presents a significant barrier (Sarre and Georges 2009). With the ongoing declines in wildlife populations occurring at a global scale, it is becoming increasingly crucial to recognize the practical implementation of genetics in wildlife conservation. Additional concerted efforts and coordinated collaboration with focused questions between evolutionary genetics and wildlife ecology are needed to achieve conservation goals.

References

Aithal GP, Day CP, Kesteven PJ, Daly AK (1999) Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. Lancet 353:717-719

Albert CA, Wilson LK, Mineau P, Trudeau S, Elliott JE (2010) Anticoagulant rodenticides in three owl species from western Canada, 1988-2003. Arch Environ Contam Toxicol 58:451-459

Andrusiak, LA, Cheng KM (1997) Breeding biology of the Barn Owl (Tyto alba pratincola) in the Lower Mainland of British Columbia. In: Duncan DR, Johnson DH, and Nicholls TH (eds.) Proceedings of the Second International Symposium on the Biology and Conservation of Owls of the Northern Hemisphere. Winnipeg MB. USDA Forest Service General Technical Report NC 190, pp 38-45

Antoniazza S, Burri R, Fumagalli L, Goudet J, Roulin A (2009) Local adaptation maintains clinal variation in melanin-based coloration of european barn owls (*Tyto alba*). Evolution 64:1944-1954

Baader M, Gnerre C, John J (2002) Transcriptional activation of Cytochrome P450 CYP2C45 by drugs is mediated by the chicken xenobiotic receptor (CXR) interacting with a phenobarbital response enhancer unit. J Biol Chem 277:15647-15653.

Barrowclough GF, Groth JG, Mertz LA, Gutierrez RJ (2006) Genetic structure of mexican spotted owl (*Strix occidentalis lucida*) populations in a fragmented landscape. Auk 123:1090-1102

Bandelt H, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37-48

Belfiore NM, Anderson SL (2001) Effects of contaminants on genetic patterns in aquatic organisms: a review. Mutat Res 489:97-122

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Statist Soc Ser B 57:289-300

Bickham JW (2011) The four cornerstones of Evolutionary Toxicology. Ecotoxicology 20:497-502

BirdLife International (2008) A range of threats drives declines in bird populations. Available http://www.birdlife.org/datazone/sowb/casestudy/120. Accessed date: Aug 22, 2014

Birky-Jr CW, Fuerst P, Maruyama T (1989) Organelle gene diversity under migration, mutation and equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and

comparison to nuclear genes. Genetics 121:613-627

Bouzat JL, Cheng HH, Lewin HA, Westemeier RL, Brawn JD, Paige KN (2008) Genetic evaluation of a demographic bottleneck in the greater prairie chicken. Conserv Biol 12:836-843

Buckle AP, Prescott CV, Ward KJ (1994) Resistance to the first and second generation anticoagulant rodenticides - a new perspective. In: Halverson WS, Marsh RE (eds.) Proceedings of the sixteenth vertebrate pest conference, pp 138-144

Buckley RC, Knedlhans SB (1986) Beachcomber biogeography: interception of dispersing propagules by islands. J Biogeogr 13.69-70.

Burri R, Niculita-Hirzel H, Roulin A, Fumagalli L (2008) Isolation and characterization of 21 microsatellite markers in the barn owl (*Tyto alba*). Mol Ecol Res 8:977-979

Bush KL, Dyte CK, Moynahan BJ, Aldridge CL, Sauls HS, Battazzo AM, Walker BL, Doherty KE, Tack J, Carlson J, Eslinger D, Nicholson J, Boyce MS, Naugle DE, Paszkowski CA, Coltman DW (2011) Population structure and genetic diversity of greater sage-grouse (*Centrocercus urophasianus*) in fragmented landscapes at the northern edge of their range. Conserv Genet 12:527-542

Caizergues A, Ratti O, Helle P, Rotelli L, Ellison L, Rasplus J (2003) Population genetic structure of male black grouse (*Tetrao tetrix L.*) in fragmented vs. continuous landscapes. Mol Ecol 12:2297-2305

Calvignac-Spencer S, Merkel K, Jutzner N, Kuhl H, Boesch C, Kappeler PM, Metzger S, Schubert G, Leendertz FH (2013) Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. Mol Ecol 22:915-924

Campbell EC, Campbell RW (1983) Status report on the common barn-owl (*Tyto alba*) in Canada. Report to British Columbia Ministry of Environment, Fish and Wildlife Branch, and Committee on the Status of Endangered Wildlife in Canada

Cegelski CC, Waits LP, Anderson NJ (2003) Assessing population structure and gene flow in Montana wolverines (*Gulo gull*) using assignment-based approaches. Mol Ecol 12:2907-2918

COSEWIC (2010) COSEWIC assessment and status report on the barn owl *Tyto alba* (Eastern population and Western population) in Canada. Committee on the Status of Endangered Wildlife in Canada. Ottawa. xiv + 34 pp

Cox P, Smith RH (1992) Rodenticide ecotoxicology: pre-lethal effects of anticoagulants on rat behaviour. In: Borreco JE, Marsh RE (eds.) Proceedings to the 15th vertebrate pesticide conference. University of California, David, California, USA. pp 165-170

CYP2C9 allele nomenclature. Human Cytochrome P450 (CYP) Allele Nomenclature

Committee Website. Available from: http://www.cypalleles.ki.se/cyp2c9.htm. Accessed Oct 10, 2014

Danielson PB (2002) The cytochrome P450 superfamily: Biochemistry, evolution and drug metabolism in humans. Curr Drug Met 3:561-597

Dixon JD, Oli MK, Wooten MC, Eason TH, McCown JW, Paetkau D (2006) Effectiveness of a regional corridor in connecting two florida black bear populations. Conserv Biol 20: 155-162

Dove CJ, Rotzel NC, Heacker M, Weigt LA (2007) Using DNA barcodes to identify bird species involved in birdstrikes. J Wildlife Manag 72:1231-1236.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359-361

Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. Mol Ecol 17:1170-1188

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792-1797

Elliott JE, Hindmarch S, Albert CA, Emery J, Mineau P, Maisonneuve F (2014) Exposure pathways of anticoagulant rodenticides to nontarget wildlife. Environ Monit Assess 186:895-906

Fellers GM, Drost CA (1991) Ecology of the island night lizard, *Xantusia riversiana*, on Santa Brabara Island, California. Herpetol Monogr 5:25-78

Frankel OH, Soule Me (1981) Conservation and evolution. Cambridge University Press, Cambridge, UK

Frankham R (1997) Do island populations have less genetic variation than mainland populations? Heredity 78:311-327

Frosch C, Dutsov A, Zlatanova D, Valchev K, Reiners TE, Steyer K, Pfenninger M, Nowak C (2014) Noninvasive genetic assessment of brown bear population structure in Bulgarian mountain regions. Mamm Biol 79:268-276

Funk CW, Forsman ED, Johnson M, Mullins TD, Haig SM (2008) Evidence for recent population bottlenecks in northern spotted owls (*Strix occidentalis caurina*). Conserv Gene 11:1013-1021

Guengerich FP (2008) Cytochrome P450 and chemical toxicology. Chem Res Toxicol 21:70-83

Guertin DA, Ben-David M, Harestad AS, Elliott JE (2012) Fecal genotyping reveals demographic variation in river otters inhabiting a contaminated environment. J Wildl Manage 76:1540-1550.

Guo Y, Zhang Y, Wang Y, Chen X, Si D, Zhong D, Fawcett Jp, Zhou H (2005) Role of CYP2C9 and its variants (CYP2C9*3 and CYP2C9*13) in the metabolism of lornoxicam in humans

Haig SM, Mullins TD, Forsman ED (2004) Subspecific relationships and genetic structure in the spotted owl. Conserv Genet 5:683-705

Henikoff S, Henikoff JG (1992) Amino acid substitution matrices from protein blocks. Proc Natl Acd Sci 89-10915-10919

Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM, Rettie AE (2002) Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. J Am Med Assoc 287:1690-1698

Hindmarch S, Krebs EA, Elliott JE, Green DJ (2012) Do landscape features predict the presence of barn owls in a changing agricultural landscape? Landscape Urban Plan 107:255-262

Hoffman AA, Blows MW (1994) Species borders: ecological and evolutionary perspectives. Trends Ecol Evol 9:223-227

Hogan FE, Campbell C, Harrison KA, Milledge D, Cooke R (2013) Molecular data contradicts historical records and cautions translocation of the Lord Howe Island masked owl. Biol Cons 159:313-320

Hogan FE, Cooke R, Burridge CP, Norman JA (2008) Optimizing the use of shed feathers for genetic analysis. Mol Ecol Res 8:561-567

Hsieh, HW, Huang LH, Tsai LC, Liu CL, Kuo YC, Hsiao C, Linacre A, Linacre L, James CI (2006) Species identification of *Kachuga tecta* using the cytochrome b gene. J Forensic Sci 51:52-56

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Res 9:1322-1332

Hull JM, Keane JJ, Savage WK, Godwin SA, Shafer JA, Jepsen EP, Gerhardt R, Stermer C, Ernest HB (2010) Range-wide genetic differentiation among North American great gray owls (*Strix nebulas*) reveals a distinct lineage restricted to the Sierra Nevada, California. Mol Phylogen and Evol 56:212-221

Hudson RR, Boos DD, Kaplan NL (1992) A statistical test for detecting geographic subdivision. Mol Biol Evol 9:138-151

International Union for Conservation of Nature (2009) More critically endangered birds on IUCN red list. Available: http://www.iucn.org/?3159/More-Critically-Endangered-birds-on-IUCN-Red-List-than-ever. Accessed date: Aug 22. 2014

Jetz W, Thomas GH, Joy JB, Redding DW, Hartmann K, Mooers AO (2014) Global distribution and conservation of evolutionary distinctness in birds. Current Biology 24:919-930

Ioannides C, Lewis DF (2004) Cytochromes P450 in the bioactivation of chemicals. Curr Top Med Chem 4:1767-1788

Kalinowski S (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. Mol Ecol Notes 5:187-189.

Karlgren M, Miura S, Ingelman-Sundberg M (2005) Novel extrahepatic cytochrome P450s. Toxicol Appl Pharmacol 207:57-61

King BP, Khan TI, Aithal GP, Kamali F, Daly AK (2004) Upstream and coding region CYP2C9 polymorphisms: correlation with warfarin dose and metabolism. Pharmogenetics 14:813-822

Kubota A, Stegeman JJ, Goldstone JV, Nelson DR, Kim E, Tanabe S, Iwata H (2010) Cytochrome P450 CYP2 genes in the common cormorant: evolutionary relationships with 130 diapsid CYP2 clan sequences and chemical effects on their expression. Comp Biochem Physiol C Toxicol Pharmacol 153:280-289

Lee M, Borgiani P, Johansson I, Oteri F, Mkrychian S, Falconi M, Ingelman-Sundberg M (2014) High warfarin sensitivity in carriers of CYP2C9*35 is determined by the impaired interaction with P450 oxidoreductase. Pharmacogenomics J 14:343-349

Leigh J (2014) PopART. http://popart.otago.ac.nz. Accessed 5 April 2014

Levins R (1969) Some demographic and genetic consequences of environmental heterogeneity for biological control. Bull Entomol Soc Am 15:237-240

Li T, Lange LA, Li X, Susswein L, Bryant B, Malone R, Lange EM, Huang TY, Stafford DW, Evans JP (2006) Polymorphisms in the VKORC1 gene are strongly associated with warfarin dosage requirements in patients receiving anticoagulation. J Med Genet 43:740-744

Maekawa K, Fukushima-Uesaka H, Tohkin M, Masegawa R, Kajio H, Kuzuya N, Yasuda K, Jawamoto M, Jamatani N, Suzuki K, Tanagawa T, Saito Y, Sawada J (2006) Four novel defective alleles and comprehensive haplotype analysis of CYP2C9 in Japanese. Pharmacogenet Genomics 16:497-514

Manel S, Berthier P, Luikart G (2002) Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. Conserv Biol 16:650-659

Manthey JD, Klicka JD, Spellman GM (2011) Cryptic diversity in a widespread North American songbird: Phylogeography of the brown creeper (*Certhia americana*). Mol Phylogenet Evol 58:502-512

Majewski J, Ott J (2003) Amino acid substitutions in the human genome: evolutionary implications of single nucleotide polymorphisms. Gene 305:167-173

Marti CD (1999) Natal and breeding dispersal in barn owls. J Raptor Res 33:181-189

Marti CD, Poole AF, Bevier LR (2005) Barn Owl (*Tyto alba*). In: Poole A (ed.) The Birds of North America, No. 1. The Birds of North America Online, Ithaca, New York. http://bna.birds.cornell.edu/. Accessed 17 May 2014

McLarty J (1995) Genetic variation in the North American barn owl (*Tyto alba*) populations using DNA fingerprinting. MSc Thesis, University of British Columbia, Vancouver, British Columbia

Meehan AP. Rats and mice: their biology and control (1984) East Grinstead, Sussex, UK:Rentokil Limited

Meek MR, Burman PJ, Nowakowski M, Sparks TH, Burman NJ (2003) Barn owl release in lowland southern England - a twenty-one year study. Biological Conservation 109:271-282

Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms Mol Ecol Notes 4:792-794

Meulenbelt I, Droog S, Trommelen GJ, Boonsma DI, Slagboom PE (1995) High-yield noninvasive human genomic DNA isolation method for genetic studies in geographically dispersed families and populations. Am J Hum Genet 57:1252-1254

Meunier B, de Visser SP, Shaik S (2004) Mechanism of oxidation reactions catalyzed by Cytochrome P450 enzymes. Chem Rev 104:3947-3980

Millus SA, Stapp P, Martin P (2007) Experimental control of a native predator may improve breeding success of a threatened seabirds in the California Channel Islands. Biol Conserv 138:484-492

Moritz C (1994) Defining 'Evolutionarily Significant Units" for conservation. Trends Ecol Evol 9:373-375

Murray M (2011) Anticoagulant rodenticide exposure and toxicosis in four species of birds of prey presented to a wildlife clinic in Massachusetts, 2006-2010. J Zoo Wildlife Med 42:88-97

Mueller GC, Miller JA (1948) The metabolism of 4-dimethylaminozobenzene by rat liver

homogenates. J Biol Chem 176:535-544

Nahar R, Dube D, Parakh R, Deb R, Saxena R, Singh TP, Verma IC (2013) Implifcation of novel CYP2C9*57 (p.Asn204His) variant in coumarin hypersensitivity. Thrombosis Res 131:535-539

NatureServe. 2014. NatureServe Web Service. Arlington, VA. U.S.A. Available http://services.natureserve.org. Accessed date: Aug 22, 2014

Nebert DW, Russell DW (2002) Clinical importance of the cytochromes P450. Lancet 360:1155-1162

Nelson DR (2009) The cytochrome p450 homepage. Hum Genomics 4:59-65

Newton I, Dale L, Finnie JK, Freestone P, Wright J, Wyatt C, Wyllie I (1998) Wildlife and pollution: 1997/98 Annual Report. Joint Nature Conservation Committee Reprot, No. 285

Newton I, Shore RF, Wyllie I, Birks S, Dale L (1999) Empirical evidence of side-effects of rodenticides on some predatory birds and mammals. In: Cowan DP, Feare CJ (eds.) Advances in vertebrate pest management. Filander Verlag, Furth, pp 347-367

Nur N, Harvey AL, Thomsen SK, Bradley R, Jahncke J (2013) Modelling the population-level impacts of barn owls on scripps's murrelet population trends on Santa Barbara Island. Unpublished report to the National Fish and Wildlife Foundation. Point Blue Conservation Science, Petaluma, California. PRBO contribution Number 1969

Oyler-McCance SJ, Kahn NW, Burnham KP, Braun CE, Quinn TW (1999) A population genetic comparison of large- and small-bodied sage grouse in Colorado using microsatellite and mitochondrial DNA markers. Mol Ecol 8:1457-1465

Paradis E, Baillie R, Sutherland WJ, Gregory RD (1998) Patterns of natal and breeding dispersal in birds. J Anim Ecol 67:518-536

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. Mol Ecol 4:347-354

Paetkau D, Slade R, Burdens M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a stimulation-based exploration of accuracy and power. Mol Ecol 13:55-65

Pelz H, Kohn M (2011) Impacts of resistance to anticoagulant rodenticides in rats and house mice on the fficacy of control measures. Report to US Environmental Protection Agency, Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel.

Pfau RS, McBee K, Van Den Bussche RA (2001) Genetic diversity of the major

histocompatibility complex of cotton rats (*Sigmodon hispidus*) inhabiting an oil refinery complex. Environ Toxicol Chem 20:2224-2228

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945-959

Proudfoot GA, Gehlbach FR, Honeycutt RL (2007) Mitochondrial DNA variation and phylogeography of the eastern and western screech-owls. Condor 109:617-628

Pruitt K, Brown G, Tatusova T, Maglott D (2002). In: McEntyre J, Ostell J, editors. The NCBI Handbook [Internet]. Bethesda (MD): National Center for Biotechnology Information (US). Chapter 18. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21091/

Rattner BA, Horak KE, Lazarus RS, Goldade DA, Johnston JJ (2014) Toxicokinetics and coagulopathy threshold of the rodenticide diphacinone in eastern screech-owls (*Megascops asio*). Environ Toxicol Chem 33:74-81

Rousset F (2008) GENEPOP '007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8:103-106

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496-2497

Rush AC, Cannings RJ, Irwin DE (2009) Analysis of multilocus DNA reveals hybridization in a contact zone between *Empidonax* flycatchers. J Avian Biol 40:614-624

Saengtienchai A, Ikenaka Y, Watanabe K, Ishida T, Ishizuka M (2010) Comparative metabolism of warfarin in rats and chickens. Poultry Sci 90:2775:2781

Sarre SD, Georges A (2009) Genetics in conservation and wildlife management: a revolution since Caughley. Wildlife Res 36:70-80

Sconce EA, Khan TI, Wynne HA, Avery P, Monkhouse L, King BP, Wood P, Kesteven P, Daly AK, Kamali F (2005) The impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. Blood 106:2329-2333

Sorenson MD, Ast JC, Dimcheff DE, Yuri T, Mindell DP (1999) Primers for a PCR-based approach to mitochondrial genome sequencing in birds and other vertebrates. Mol Phylogenet Evol 12:105-114

Spellman GM, Riddle B, Klicka J (2007) Phylogeography of the mountain chickadee (*Poecile gamble*): diversification, introgression, and expansion in response to Quaternary climate change. Mol Ecol 16:1055-1068

Stewart P (1952) Winter mortality of barn owls in central Ohio. Wilson Bull 64:164-166

Sugimoto T, Nagata J, Aramilev VV, Belozor A, Higashi S, McCullough DR (2006) Species and sex identification from faecal samples of sympatric carnivores, Amur leopard and Siberian tiger, in the Russian Far East. Conserv Genet 7:799-802

Taylor I (1994) Barn Owls. Cambridge University Press, Cambridge, UK

Thomas PJ, Mineau P, Shore RF, Champoux L, Martin PA, Wilson LK, Fitzgerald G, Elliott JE (2011) Second generation anticoagulant rodenticides in predatory birds: Probabilistic characterisation of toxic liver concentrations and implications for predatory bird populations in Canada. Environ Intern 37:914-920

Thomsen SK, Kroeger CE, Bloom PH, Harvey AL. 2014. Space use and home range size of barn owls on Santa Barbara Isaldn. Monogr West N Am Naturalist *In press*

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535-538

Vandenbroucke V, Bousuqet-Melou A, De Backer P, Croubles S (2008) Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. J Vet Pharmacol Ther 31: 437:445

Vucetich JA, Waite TA (2003) Spatial patterns of demography and genetic processes across the species' range: Null hypotheses for landscape conservation genetics. Conserv Genet 4:639-645

Walker LA, Chaplow JS, Llewellyn NR, Pereira MG, Potter ED, Sainsbury AW, Shore RF (2013) Anticoagulant rodenticides in predatory birds 2011: a Predatory Bird Monitoring Scheme (PBMS) report. Centre for Ecology and Hydrology, Lancaster, UK. pp 1-29

Walstrom VW, Klicka JD, Spellman GM (2012) Speciation in the white-breasted nuthatch (*Sitta carolinensis*): a multilocus perspective. Mol Ecol 21:907-920

Warren W, Hillier L, Mardis E, Wilson R (2005) Additional sequencing of the chicken genome. http://www.genome/gov/Pages/Research/Sequencing/SeqProposals/ChickenfinishingSeq.pdf. Accessed November 8, 2014.

Wasser SK, Hunt KE (2005) Noninvasive measures of reproductive function and disturbance in the barred owl, great horned owl, and northern spotted owl. Ann N Y Acad Sci 1046:109-137

Watanabe KP, Saengtienchai A, Tanaka K, Ikenaka Y, Ishizuka M (2010) Comparison of warfarin sensitivity between rat and bird species. Comp Biochem Physiol., Part C 152:114-119

Watanabe KP, Kawai YK, Ikenaka Y, Kawata M, Ikushiro S, Sakaki T, Ishizuka M (2013) Avian

Cytochrome P450 (CYP) 1-3 family genes: isoforms, evolutionary relationships, and mRNA expression in chicken liver. PLoS ONE 8: e75689

Wink M, El-Sayed A, Sauer-Gurth H, Gonzelez J (2009) Molecular phylogeny of owls (strigiformes) inferred from DNA sequences of the mitochondrial cytochrome *b* and the nuclear *RAG-1* gene. Ardea 97:581-591

Wisely SM, Buskirk SW, Russell GA, Aubry KB, Zielinski WJ (2004) Genetic diversity and structure of the fisher (*Martes pennant*) in a peninsular and peripheral metapopulation. J Mammal 85:640-648

Appendices

Appendix A – Additional necropsy information of the 13 barn owl samples with their CYP2C45 gene sequenced

ID #	Date Found	Location in BC	Age	Sex	Comments
L12-555	21-Dec-11	Surrey	Adult	Female	Body mass 444g, good body condition, peri-renal hemorrhage, patchy hemorrhage in lungs, no fractures, bruising over right hip
L12-559	28-Nov-10	New Westminster	Nestling	Male	Body mass 396 g, poor body condition, pale, gastric and intestinal hemorrhage
L12-709	11-Feb-12	Langley	Adult	Male	Body mass 355g, poor body condition, emaciated, dark intestinal content (blood?), blood in stomach, full gall bladder
L12-736	15-Oct-12	Langley	Nestling	Male	Body mass 355 g, poor body condition, hemorrhage in stomach, pale, no fractures
L12-753	13-Nov-12	Richmond	Adult	Female	Body mass 535 g, poor body condition, gastric hemorrhage, clear respiratory airway, no fractures
L12-727	28-Sep-12	Delta	Adult	Male	Body mass 440 g, fair body condition, hemopericardium, pulmonary hemorrhage, no fractures, cardiac contusion, GIT - vole
L12-737	30-Sep-12	Langley	Adult	Female	Body mass 420 g, poor body condition, chronic bumblefoot on left foot, dry necrotic ulcerated foot pad
L12-738	7-Feb-12	Langley	Adult	Male	Body mass 510 g, good body condition, fracture distal left femur, thoracic pulmonary hemorrhage, lacerated liver, GIT shrew
L12-558	15-Jan-12	Delta	Nestling	Female	Body mass 371 g, poor body condition, pale, hemorrhage in stomach and intestines, tapeworms, pulmonary nematodes
L12-732	4-Nov-12	Abbotsford	Adult	Female	Body mass 470 g, fair body condition, multiple fracture of both wings, pulmonary hemorrhage
L12-733	11-Oct-12	Chilliwack	Adult	Female	Body mass 495 g, fair body condition, fracture right distal humorous, thoracic hemorrhage
L12-752	10-Nov-12	Richmond	Adult	Male	Body mass 420 g, poor body condition, clear respiratory tract, no fractures or hemorrhage
L12-949	11-Nov-12	Richmond	Adult	Female	Poor body condition, gastric hemorrhage, dark intestinal content, fractured pelvis, dislocated right hip, swollen right foot

Appendix B – SNPs identified in the non-coding regions (introns) of the CYP2C45 gene and their associated nucleotide positions (starting from the start codon of exon 1). Highlighted in red are individuals categorized as “low AR tolerance”, and blue are “high AR tolerance”.

	Nucleotide position								
	459	510	1023	1875	1913	1979	2662	3188	3223
L12-558	R	Y	R	G	C	Y	K	Y	G
L12-559	R	Y	G	G	C	T	K	Y	R
L12-709	R	T	G	G	C	T	K	C	G
L12-736	A	C	A	G	C	T	G	T	G
L12-753	G	T	G	G	C	T	T	C	R
L12-727	G	T	G	R	A	T	K	C	G
L12-737	A	Y	R	G	C	T	G	T	G
L12-738	R	T	G	G	C	T	K	C	R
L12-555	R	Y	G	G	C	T	K	Y	R
L12-732	G	T	G	G	A	T	K	C	G
L12-733	R	C	A	G	C	T	G	Y	G
L12-752	G	T	G	R	C	Y	T	C	G
L12-949	R	Y	R	R	C	T	K	Y	G