Title

Critical Effects of Urbanization on a Charismatic Carnivore: Genetic Change, Disease and Toxicant Exposure, and Disease Susceptibility in Bobcat Populations in an Urban, Fragmented Landscape

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Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Critical Effects of Urbanization on a Charismatic Carnivore: Genetic Change, Disease and Toxicant Exposure, and Disease Susceptibility in Bobcat Populations in an Urban, Fragmented Landscape

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biology

by

Laurel Elizabeth Klein Serieys

2014

ABSTRACT OF THE DISSERTATION

Critical Effects of Urbanization on a Charismatic Carnivore:
Genetic Change, Disease and Toxicant Exposure, and Disease Susceptibility
in Bobcat Populations in an Urban, Fragmented Landscape

by

Laurel Elizabeth Klein Serieys
Doctor of Philosophy in Biology
University of California, Los Angeles, 2014
Professor Robert K. Wayne, Chair

Urbanization has profound ecological impacts that reach beyond city boundaries. Obvious ecological consequences of urbanization include habitat loss and fragmentation. Anthropogenic barriers reduce habitat connectivity, impede gene flow between populations and accelerate the loss of genetic diversity in populations due to drift. Urbanization may have also cryptic consequences such as the effects of human-introduced toxicants on wildlife populations. Toxicants are a leading cause of population decline for a variety of animal species worldwide and may directly threaten animal populations by causing direct mortalities, or indirectly through sublethal, chronic effects such as reproductive impairment, decreased immune competence, and

increased disease susceptibility or emergence. If population-level impacts occur as a result of toxicant exposure, genetic consequences may also accompany reduced population sizes and connectivity. These include inbreeding depression that may increase the probability of population extinction and the loss of adaptive potential that reduces the ability of populations to respond to novel selection regimes. Overall, urbanization presents wildlife with many novel stressors to which they must adapt or perish. Urbanization is increasing at an unprecedented pace; understanding both the obvious and the cryptic threats to wild animal populations persisting near urban areas will be vital to promoting conservation and the maintenance of global biodiversity.

To address the consequences of urbanization on wildlife populations, I focused on a well-studied population of bobcats (*Lynx rufus*) living in and around Santa Monica Mountains National Recreation Area (SMMNRA). This region comprises a collection of protected park areas near downtown Los Angeles. Bobcats inhabiting SMMNRA have been monitored by National Park Service (NPS) biologists since 1996. Within a localized region of SMMNRA, the NPS has demonstrated that a major freeway (US-101) acts not only as a barrier to movement for bobcat and coyote (*Canis latrans*) populations, but potentially also as a social barrier. Further, from 2002-2005, a notoedric mange epizootic associated with secondary anticoagulant rat poison exposure was the greatest source of mortality for bobcats. During this period, the annual survival rate for radio-collared animals fell by > 50% and in 2003 the mange mortality rate reached a high of 51%. Long-term samples were collected from this population from 1996-2012, allowing the rare opportunity to make direct comparisons before, during, and after the population decline.

Using these data as a foundation, my research focused on three main objectives. First, I characterized neutral and adaptively relevant genetic diversity in bobcat populations across SMMNRA in both fragmented urban and protected natural areas. Second, I examined anticoagulant rodenticide exposure in bobcats across southern California, contrasting seasonal,

demographic and spatial risk factors in both natural and urbanized areas. Third, I characterized physiological and immunological parameters in bobcats across SMMNRA to evaluate the effects of disease and toxicant exposure on bobcat health in an urban, fragmented landscape.

I found that two freeways are significant barriers to gene flow. Further, the 3-year disease epizootic, associated with secondary anticoagulant rodenticide exposure, caused a population bottleneck that led to significant genetic differentiation pre- and post-disease populations that was greater than that between populations separated by major freeways for > 60 years. However, balancing selection acted on immune-linked loci during the epizootic, maintaining variation at functional regions. With respect to anticoagulant rodenticide exposure, I detected high prevalence of exposure (89%, liver; 39%, blood) and found that for individuals with paired liver and blood data (N = 64), 92% were exposed most frequently to ≥ 3 compounds. Prevalence and the amounts of contaminants were associated with human activities that included commercial, residential, and agricultural development. I found a strong association between AR exposure to ≥ 0.25 ppm or ≥ 2 compounds and an ectoparasitic disease, notoedric mange. Finally, I observed that AR exposure has both immune stimulatory and suppressive effects that may explain increased bobcat susceptibility to notoedric mange as a result of chronic exposure to anticoagulant rodenticides. Bobcats exposed to ARs had elevated lymphocyte, and specifically B cell counts, and decreased percentages of neutrophils. Overall, these data highlight that even for free-ranging animals that are considered relatively adaptable to urbanization, habitat fragmentation and toxicant exposure can have profound population level effects that threaten the long-term stability of wildlife populations in an increasingly urbanized landscape.

The dissertation of Laurel Elizabeth Klein Serieys is approved.

James O. Lloyd-Smith

Christina Uittenbogaart

Seth P.D. Riley

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2014

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ACKNOWLEDGMENTS

First and foremost I express my deep thanks to both Drs. Seth P.D. Riley and Robert K. Wayne, for their unwavering mentorship, support, encouragement, and enthusiasm throughout my studies at UCLA. Seth recognized early the contribution I could make to ongoing research in the Santa Monica Mountains, and facilitated my entry to a world of research I'd long dreamed about-studying the impacts of urbanization on carnivore populations. Bob, had faith that a field biologist could learn to answer new and exciting questions in a free-ranging carnivore population using the laboratory techniques necessary to bridge the field research with molecular and immunological techniques. Together, Seth and Bob encouraged me to be creative and independent in the design of my study, and yet were always available to assist me when I needed their guidance.

My thesis committee, comprised of Drs. Christel Uittenbogaart, Jamie Lloyd-Smith, and Thomas Smith, also provided invaluable support during many stages of my research. In particular, I would like to acknowledge Christel, who was always available for both academic and personal support, and who welcomed me as a valued member of her lab. When searching for an immunology lab to collaborate with, I quickly discovered that I would have to find a principal investigator willing to think outside the box, dare to allow non-human samples into their lab space, and have faith that a field biologist could develop the laboratory skills to explore immunology in a wild animal population. I thank Christel for her open-mind and willingness to branch out from human immunology to help me explore bobcat immunology.

My parents and family were enduring, faithful and proud supporters during my graduate school experience. My mother, Roxanna Klein, is especially enthusiastic about my work and always eager for updates. I am thrilled that I had the chance to share time in the field at bobcat and mountain lion captures with both of my parents. Mom and dad sought ways to ease some of

the logistical challenges of my research by sponsoring some of the critical supplies needed to get started with my field research.

During the past eight years, I have developed new special friendships, and strengthened bonds with others. I was fortunate enough to meet both Devaughn Fraser and Tiffany Armenta when they became my field assistants. They transitioned from field assistants to colleagues and good friends when they began their own doctoral program in the Wayne Lab. I am profoundly grateful to have had the chance to work with them and have their friendship, particularly during the transition from graduate student to postdoctoral researcher. I also thank my good friend Christi Garner, who has always encouraged me to make time for myself and live a healthy lifestyle, while inspiring me to be daring and pursue adventure.

Many collaborators and assistants provided critical support to this project. Joanne Moriarty, Cathy Schoonmaker, Jeff Sikich, Justin L. Brown, Dr. Erin Boydston and Lisa Lyren were invaluable assets to the field component of this study. Joanne, in particular, played a key role in this project; she trained me in capture and handling techniques, was available to provide additional assistance in the field when needed, and was enthusiastic about each of my research questions. Erin and Lisa generously provided supplies that increased my field productivity and data that enhanced the value of this study. Erin provided much appreciated mentorship and support throughout multiple phases of this research. My collaborators Janet Foley, Scott Carver, and Sue VandeWoude were excellent sounding boards and mentors for the disease component of this study. Without Marta Epeldegui, I would not have been successful with the development of immunological assays, and so I am extremely grateful that she dedicated precious time to assist me in the lab. I also have Mary Ann Hausner, Ingrid Schmid, Janet Dow, Larry and Patty Hutlin, Scott Weldy, and Kristi Krause to thank for the help they provided with the development of my immune assays. Ryan Harrigan was always patient with my many data analysis questions and

always gave me a reason to laugh– I feel lucky to have shared a work arena with him. Lab assistants Amanda Lea, Victoria Hsu and Adam Uchimoto contributed numerous hours to help me complete lab work, while Craig Reddell, Jason Warner, Tiffany Armenta, and Devaugh Fraser were critical members of my field team.

I would like to thank my many funding sources. The Santa Monica Bay Audubon Society (SMBAS) provided financial support for my work since my research began and members of the society were dedicated followers through the development of my research. Dan and Susan Gottlieb and the G2 gallery were also eager supporters of my work, and taught me to think creatively when rallying support for my project. Barry Rowan, shared his exceptional photos of wild bobcats, providing me the opportunity to work with the G2 gallery to raise awareness about the challenges to the persistence of carnivore populations in Los Angeles. Julie Newsome has been an enthusiastic supporter of my website, UrbanCarnivores.com, and encouraged me to take creative tactics to fundraising. Joel and Kian Schulman took exceptional steps to communicate the findings of my rodenticide research with a broad public audience, and because of them, I learned that the efforts of few could lead to big changes in the way that people relate to their natural surroundings. I also express deep gratitude to Art Eck, for his enthusiastic support of my website and for managing a research account on my behalf, despite the considerable work it created for him.

I dedicate this work to my good friend Eric Charles York, with whom I deeply wish I could share the results of my many years of work on urban bobcats. Eric taught me many tricks of the trapping trade during our time in the field together. I will always cherish the short time I was able to spend with him, and our shared appreciation for the outdoors, adventure, and wildlife. As I’ve grown through the process of graduate school, I have realized that he saw in me the person I

have become. He lived the life he'd imagined, and in doing so, he encouraged me to go confidently in the direction of my dreams.

Most importantly I express my profound and deep gratitude to my devoted husband, Nicolas Serieys. He has supported me throughout the many phases of my research by providing a stable foundation of emotional support. Always patient with my busy schedule. Nico understands my passion for science and wildlife, and gives me the space to pursue those passions. Nico is constant source of love and support, regularly reminding me to take time for myself to recharge my batteries. He has aided that process as well with many a good meal and weekend trips. By example, Nico has shown me that there is life outside of work, and that we must make time to enjoy it. In so doing, we can more effectively do the important work that we all share.

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Serieys, L.E.K., Boydston, E., Lyren, L.M., Poppgenga, R.H., Wayne, R.K., and S.P.D. Riley 2012. Anticoagulant rodenticides: Is a rodent poison a big problem for bobcats in an urban, fragmented landscape? *The Annual Wildlife Society Meeting*, Portland, Oregon. Talk.

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Klein, L., Riley, S.P.D., Foley, J., Owens, S., Boydston, E.E., Lyren, L.M., Crooks, K., and R.K. Wayne. 2011. Notoedric mange in bobcats in Southern California. *TWS, The Western Section Conference*, Irvine, California. Invited Talk.

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CHAPTER 1

Disease and freeways drive genetic change in urban bobcat populations

ABSTRACT

Urbanization profoundly impacts animal populations by causing isolation, increased susceptibility to disease, and exposure to toxicants. The genetic effects of isolation include reduced effective population size, increased population substructure, and decreased adaptive potential. Although the consequences of urbanization are increasingly Ill known, to our knowledge, no previous studies have examined how urbanization affects genetic variation at both neutral and functional loci. I investigate the influence that urbanization and a disease epizootic had on the population genetics of bobcats (*Lynx rufus*) distributed across a highly fragmented urban landscape. I genotype more than 300 bobcats, sampled from 1996-2012, for variation at nine neutral and seven immune gene-linked microsatellite loci. I predict that urbanization and disease shape genetic structure and diversity, and that disease enhances selection at immune genes. I find that two freeways are significant barriers to gene flow. Further, a 3-year disease epizootic (associated with secondary anticoagulant rat poison exposure) caused a population bottleneck that led to significant genetic differentiation pre- and post-disease populations that was greater than that between populations separated by major freeways for > 60 years. However, balancing selection acted on immune-linked loci during the epizootic, maintaining variation at functional regions. Conservation assessments need to assay loci that are potentially under selection in order to better preserve the adaptive potential of populations at the urban-wildland interface. Further, inter-connected regions that contain appropriate habitat for wildlife will be critical to the long-term viability of animal populations in urban landscapes.

INTRODUCTION

Anthropogenic barriers reduce habitat connectivity, impeding gene flow between populations and accelerating the loss of genetic diversity due to drift (Hedrick 2005; Epps *et al.* 2005; Riley *et al.* 2006; Keyghobadi 2007; Delaney *et al.* 2010; Lee *et al.* 2012). In addition, urbanization may contribute to increased disease exposure (Bradley & Altizer 2007) that can cause precipitous population declines (LoGiudice 2003; Riley *et al.* 2007). Genetic consequences that accompany reduced population size and connectivity include inbreeding depression that may increase the probability of population extinction (Saccheri *et al.* 1998; Coltman *et al.* 1999; Keller 2002; Spielman *et al.* 2004) and the loss of adaptive potential that reduces the ability of populations to respond to novel selection regimes (Lande 1998; Frankham 2005; Keyghobadi 2007). Yet despite recognition that urban populations are vulnerable to reduced genetic connectivity and population declines, few studies have characterized neutral or adaptively relevant genetic diversity in urban populations (Hitchings & Beebee 1997; Keyghobadi 2007).

Gene regions related to immune function are considered paradigms for the study of important adaptive genetic diversity (Piertney & Oliver 2005). The major histocompatibility complex (MHC) and toll-like receptors (TLRs) include the most polymorphic families of genes known, and these genes encode key components of the vertebrate immune system (Klein 1986; Jepson *et al.* 1997; Hill 1998; Roach *et al.* 2005; Iwasaki & Medzhitov 2010). TLRs are a critical component of innate immunity that provide non-specific protection against a variety of disease-causing organisms (Aderem & Ulevitch 2000; Janeway & Medzhitov 2002). MHC genes encode cell-surface molecules that present antigens derived from parasites or pathogens to T lymphocytes to initiate innate and adaptive immune responses (Klein 1986). Both positive and balancing selection are considered important drivers of genetic diversity at regions associated

with immune function (Bernatchez & Landry 2003; Piertney & Oliver 2005; Ferrer-Admetlla *et al.* 2008; Areal *et al.* 2011). However, studies done in free-ranging animal populations have implicated both drift and selection in shaping variation at immune-linked loci (Bernatchez & Landry 2003; Piertney & Oliver 2005; Bollmer *et al.* 2011).

To address the consequences of urbanization on wildlife population genetics, I focused on a well-studied population of bobcats (*Lynx rufus*) living in and around Santa Monica Mountains National Recreation Area (SMMNRA). This region comprises a collection of protected park areas near downtown Los Angeles. Importantly, previous research has demonstrated that this population is negatively impacted by urbanization in multiple ways, namely, through habitat fragmentation (Riley *et al.* 2006) and increased disease susceptibility associated with contaminant exposure (Riley *et al.* 2007). As a result, this population is appropriate for studying the increasingly widespread impacts of urbanization on free-ranging animal populations, the influence of habitat fragmentation on gene flow and neutral genetic diversity, and the effects of increased disease susceptibility and a consequent population decline on immune-related genetic variation.

Bobcats inhabiting SMMNRA have been monitored by National Park Service (NPS) biologists since 1996. Within a localized region of SMMNRA, the NPS has demonstrated that a major freeway (US-101) acts not only as a barrier to movement for bobcat and coyote (*Canis latrans*) populations, but potentially also as a social barrier. Specifically, the small percentage of bobcats (11.5%) and coyotes (4.5%) that successfully crossed the 101 Freeway were unlikely to establish territory and reproduce due to hypothesized territory pile-up on both sides of the freeway (Riley *et al.* 2006). As a result, carnivore populations separated by the US-101 were significantly genetically differentiated, although they were separated by < 1km (Riley *et al.*

2006). The 101 Freeway has also been shown to be an important barrier to gene flow for other species including side-blotched lizards (*Ula stansburiana*), western fence lizards (*Sceloporus occidentalis*), western skinks (*Plestiodon skiltonianus*) and wrentits (*Chamaea fasciata*) (Delaney *et al.* 2010). Taken together, these findings suggest that urbanization, and particularly major freeways, can severely interrupt gene flow in a variety of taxa.

In addition to creating barriers to dispersal and gene flow, urbanization has negatively impacted the SMMNRA bobcat population through increased disease susceptibility. From 2002-2005, a notoedric mange epizootic associated with secondary anticoagulant rat poison exposure was the greatest source of mortality for bobcats (Riley *et al.* 2007). During this period, the annual survival rate for radio-collared animals fell by > 50% and in 2003 the mange mortality rate reached a high of 51%. Long-term samples were collected from this population from 1996-2012, allowing the rare opportunity to make direct comparisons before, during, and after the population decline. Furthermore, samples were more recently collected throughout SMMNRA from populations experiencing varying levels of habitat fragmentation and isolation, allowing me to examine whether barriers to gene flow (e.g., major freeways) accelerated the genetic consequences of a population decline associated with disease.

To address the effects of urbanization on genetic diversity in a highly mobile carnivore, I genotyped approximately 300 bobcats at nine neutral and seven microsatellite loci in or near immune-related genes. Specifically, I investigated how freeways and a population decline associated with disease influenced population isolation, structure, and differentiation. In addition, I characterized the role of selection as a result of disease and genetic drift on immune-related genes in populations separated by urbanization and freeways. The use of microsatellite loci linked with immune regions enabled me to determine the influence of disease and urbanization

on a variety of immunologically important loci. Further, my use of both neutral and immune-linked microsatellite loci enabled me to determine whether habitat fragmentation, urbanization, and the disease outbreak and subsequent population decline differentially affect functional versus neutral genetic diversity.

I predicted that major urban barriers (e.g., freeways) impede gene flow and promote population isolation and genetic drift; consequently, populations separated by freeways should be highly differentiated at neutral loci. In addition, I hypothesized that the population decline associated with the disease outbreak should enhance genetic drift; thus, samples collected after the disease outbreak were expected to show reduced genetic diversity at neutral loci compared to samples collected before the disease outbreak and would be genetically differentiated from each other. With respect to immune-related loci, I expected that heterogeneity in disease prevalence and in the severity of population isolation would lead to differential patterns of selection and genetic drift. Specifically, I predicted that the population severely impacted by disease would experience directional or balancing selection at immunologically important genes, as genetic variation at these loci may confer resistance to disease (Schröder & Schumann 2005; Froeschke & Sommer 2005; Barreiro *et al.* 2009; Savage & Zamudio 2011; de Assunção-Franco *et al.* 2012). Balancing selection would be expected to maintain or enhance variation across a bottleneck whereas directional selection would reduce genetic diversity. However, for populations with a high degree of isolation or unaffected by disease, I predicted that genetic drift may be the critical driver of genetic variation at immune-linked loci.

METHODS

Study area and sample collection

Bobcats were sampled in and around Santa Monica Mountains National Recreation Area (SMMNRA, Fig. 1.1) in Southern California. The study area is comprised of more than 620 km^2, encompassing large regions of continuous habitat with minimal urban development, as well as highly fragmented areas that extend east through Los Angeles. The area straddles two major 8- to 10- lane freeways, US-route 101 and Interstate-405. US-route 101 (the Ventura Freeway) was established in 1949 and has been demonstrated by genetic analyses to be a significant geographic and social barrier for bobcat and coyote populations (Riley *et al.* 2006). Route I-405 was established in 1962 (www.cahighways.org) and is the most travelled highway in the country with more than 370,000 vehicles daily (www.fhwa.dot.gov). Many secondary roads are also found throughout SMMNRA. Human land uses within SMMNRA include commercial and residential development and altered green areas such as golf courses.

Bobcats sampled from SMMNRA were classified as belonging to one of three geographic regions: 1) highly fragmented habitat east of I-405 and including an area east of the 101 Freeway in Griffith Park sampled from 2010-2011 (E405 population); 2) a region west of the I-405 and south of the 101 Freeway (S101 population) that includes most of the Santa Monica Mountains and comprises largely undeveloped protected parkland intersected by secondary roads and pockets of urban development; and 3) an area north of the 101 Freeway (N101 population) consisting largely of habitat patches interspersed with roads and development (Fig. 1.1).

From 1996-2012, long-term bobcat sampling occurred in the N101 region, as well as in Malibu Creek State Park (Fig. 1.1, MCSP), an isolated subset of S101 as previously described (Riley *et al.* 2006). From 2002-2005, an epizootic of notoedric mange associated with secondary

anticoagulant rodenticide exposure occurred primarily north of the 101 Freeway, causing a sharp population decline during the latter half of the epizootic (Riley *et al.* 2007). Bobcats were sampled before, throughout, and after the mange epizootic, and consequently, analyses described below were primarily conducted on two putative N101 populations. The first population included animals sampled before and during the mange epizootic (N101 before/during mange) from 1996-2005. These two periods were grouped because I expected genetic consequences of the disease outbreak to occur after the population decline that primarily occurred during 2003-2005. The second population consisted of animals sampled after the mange epizootic (N101 post-mange) from 2006-2012. Thus, a total of four putative populations for this study included E405, S101, N101 before/during mange and N101 post-mange.

Sample collection across all regions consisted of blood, tissue or buccal swabs obtained by capturing animals or opportunistic tissue obtained postmortem from carcasses discovered in the study area (Table A.1, Appendix). Fresh scat samples were opportunistically collected during trapping seasons. Bobcats were captured with padded foothold traps (1996-1998) and cage and box traps (2000-2012) as in Riley *et al.* (2006). Once captured, animals were aged, sexed, weighed, measured, ear-tagged, and for a subset of animals captured by NPS biologists, they were also radio-collared. All animals were then released at the capture site. Animal capture, handling, and sample collection protocols were approved by the Office of Animal Research Oversight of the University of California, Los Angeles (Protocol ARC#2007-167-12). Protocols underwent extensive review in order to minimize animal stress and suffering. Scientific collecting permits were authorized through California Department of Fish and Wildlife (SC-9791).

Genotyping

DNA was extracted from tissue, blood, and buccal swabs using the QIAamp® DNA Mini Kits (Qiagen) and from fecal samples using QIAamp® DNA Stool Mini Kits (Qiagen) according to the manufacturer's protocols. Individuals were genotyped using nine supposed neutral dinucleotide microsatellite markers (FCA008, FCA023, FCA026, FCA043, FCA045, FCA077, FCA090, FCA096, and FCA132) developed for the domestic cat (Menotti-Raymond *et al.* 1999) and previously used in bobcat studies (Ernest *et al.* 2000; Riley *et al.* 2006). To ensure accurate genotypes of lower quality fecal DNA and to prevent contamination with higher quality tissue DNA, polymerase chain reactions (PCR) for scat samples were performed on 96-well plates separately from those used to genotype tissue samples. To ensure accurate genotype calls, fecal samples were genotyped multiple times. Heterozygous loci were confirmed with at least one additional amplification, and homozygous loci were amplified a minimum of three times. When consistent results could not be achieved for specific loci after multiple amplifications, the single-locus genotypes were treated as missing data. Any scat samples with more than two loci of missing data were excluded from analyses. Samples that had consistently poor amplification across multiple loci, or suspected contamination (amplification of > 2 alleles), were removed from further analyses.

To assess the influence of urbanization and disease on patterns of genetic diversity at regions of functional importance, samples were also genotyped at seven immune-related microsatellite loci. I chose five MHC regions found on chromosome B2 in the domestic cat (Beck *et al.* 2005) that included a Class I locus (FLA1) and four Class II loci (DRA1, DRB1, DRB3, and DRB4). Additionally, two toll-like receptor associated loci were chosen (TLR3 and TLR4) using the cat genome assembly version Felcat4 (Pontius *et al.* 2007). Previous studies

have set an empirical precedent for the use of microsatellites linked with immune regions, or other functional regions, to measure selection and fitness effects in a variety of taxa (e.g. Ammer *et al.* 1992; Ellegren *et al.* 1993; Schwaiger *et al.* 1994; Aguilar *et al.* 2004; Acevedo-Whitehouse *et al.* 2006; Santucci *et al.* 2007; Banks *et al.* 2009; Tollenaere *et al.* 2012). The reliability of microsatellite variation to indicate variation at the MHC has been verified empirically, and in the absence of selection, has similar properties to microsatellites in neutral regions (Boyce *et al.* 1997; Moghaddam *et al.* 1998; Tollenaere *et al.* 2012).

I used MSATCOMMANDER (Faircloth 2008) to design primers (Table A.2, Appendix) for the amplification of immune regions. The program enables rapid microsatellite repeat detection based on motif-matching with a specific, user-defined DNA sequence. The program also allows rapid and automated design of locus-specific primers and 5'-tailing primers. Primers designed using this program were validated with domestic cat DNA and tested for polymorphism and amplification efficacy with bobcat DNA. Patterns consistent with a single locus were observed in all amplifications.

Genotypes were obtained by PCR amplification of all 16 loci as in Riley *et al.* (2006). Briefly, PCRs utilized either a fluorescent dye-labeled forward primer, or a hybrid combination of forward primers consisting of the published forward primer with the M13F (−20) sequence (16 bp) added to the 5′end and a fluorescent dye labeled M13F (−20) primer. The unlabeled reverse primer was used in both cases. Established PCR conditions were used for the hybrid combination primer (a two-step cycle; Boutin-Ganache *et al.* 2001). Primer dye-labeling utilized BeckmanCoulter dye D4 and PCR products were sized on the BeckmanCoulter CEQ2000XL DNA Analysis System (Riley *et al.* 2006).

Validating and characterizing microsatellite data

Deviations of each locus from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENEPOP version 4.0 (Raymond & Rousset 1995). Each marker pair was tested for linkage disequilibrium within each population, and because > 100 comparisons were made, I corrected the critical value corresponding to $\alpha = 0.05$ for 104 comparisons ($\alpha = 0.0005$) (Rice 1989). Global tests of heterozygote deficit and excess were conducted for each population, as well as for each locus within populations, and because 32 tests were conducted per population, I corrected for multiple tests ($\alpha = 0.002$**)**. Genotyping error and null alleles were evaluated using MICRO-CHECKER (van Oosterhout & Hutchinson 2004) across all loci for all individuals as well as for the four putative populations. MICRO-CHECKER tests not only for the presence of null alleles, but can also distinguish between scoring errors due to large-allele dropout or stuttering.

Genetic Diversity

Observed (H_O) and expected (H_E) heterozygosities (Nei 1987) were calculated using CERVUS (Marshall *et al.* 1998). Per locus allelic richness (A_R) (Mousadik & Petit 1996) and the inbreeding coefficient (F_{IS}) were estimated using FSTAT 2.9.3 (Goudet 1995). I estimated F_{IS} significance for all loci and all populations using 560 randomizations in FSTAT 2.9.3. Data were tested for normality using the Shapiro-Wilke test. Across population differences for each measure were tested either with a Kruskal-Wallis or ANOVA, while pairwise comparisons were calculated with Mann-Whitney or *t*-tests ($\alpha = 0.05$). Because theory predicts changes in heterozygosity when a significant population decline occurs, allelic richness, observed and expected heterozygosity, and the inbreeding coefficient were also tested for differences between

the N101 before mange (1996-2001) and the N101 post-mange (2006-1012). Genetic diversity values for the N101 population before, during, and after the mange epizootic are presented in the Appendix (Table A.3). The significance of differences was tested with Wilcoxon-rank sum or *t*-tests ($\alpha = 0.05$). All across group and pairwise analyses were conducted in R (R Development Core Team, 2012).

Demography

I obtained estimates of effective population size (N_e) across populations using a linkage disequilibrium (LD) method (Waples 2006) implemented in LDNe (Waples & Do 2008), a bias-corrected estimator of effective population size. The LD method takes advantage of the principle that as effective population size decreases, genetic drift and limited reproduction generate nonrandom associations among alleles at different loci (Hill 1981; Waples 1991). To evaluate the fine-scale temporal impact of the disease outbreak on the effective population size over time, N_e for the N101 population was measured in 1-2 year increments, depending on the number of animals captured yearly such that all $N > 20$ ($\overline{N} = 26$; Table 1.1). The program is reported to perform well in non-ideal populations (e.g. skewed sex ratio, nonrandom mating, or small sample sizes). Waples (2006) found that for smaller sample sizes ($N = 10$ or 20), the performance of the program was nearly equivalent to that for sample sizes of $N \geq 30$.

I used two methods to test for evidence of a genetic bottleneck across all sampled populations and years. Using the neutral loci, I employed BOTTLENECK 1.2.02 (Cornuet & Luikart 1996; Piry *et al.* 1999) to test for significant heterozygosity excess compared to mutation-drift equilibrium expectations for a stable population. Extreme population reductions result in a disproportionate loss of rare alleles that contribute relatively little to expected

heterozygosity. Consequently, for a recently bottlenecked population, there will be a transitory excess of observed heterozygosity compared with a population at equilibrium with the same number of alleles (Cornuet & Luikart 1996). As above, I divided N101 samples into 1-2 year increments (all N > 20, $\overline{N}$ = 26). Cornuet and Luikart (1996) suggest a minimum sample size of 20 diploid individuals to achieve a reasonably high degree of power in bottleneck tests. I tested each subset for heterozygosity excess. For the MCSP population subset of S101, I also tested for heterozygosity excess during 1996-2001 (N = 30) and 2006-2012 (N = 26) to determine if a population bottleneck occurred regionally across the study area or was isolated to the N101 population. I ran 1,000 iterations of the two-phase model (TPM) recommended for microsatellite data, with a variance (σ^2_g) of 12 and 90% single-step mutations (p_s = 90%) (Rienzo 1994; Brinkmann *et al.* 1998; Piry *et al.* 1999; Garza & Williamson 2001). Recently, Peery *et al*. (2012) suggested experimenting with alternative parameters when calculating bottleneck tests, and specifically testing up p_s = 78%, because this value represents the mean estimate of single-step mutations in vertebrate studies. Thus, I also tested for heterozygote excess using this alternative parameter. To evaluate significance, I used the Wilcoxon signed-rank test recommended for polymorphic microsatellite data (Piry *et al.* 1999).

In addition, I tested for a bottleneck signature using Garza and Williamson's M-RATIO method implemented in the program M_P_VAL (Garza & Williamson 2001). The approach calculates *M*, the mean ratio of the number of alleles to range in allele size, which can be used to detect reductions in effective population size. For bottlenecked populations, the range in allele size will decrease more slowly than the number of alleles lost during a reduction in effective population size. In this case, *M* ratios will be smaller than 1.0, and for populations known to have undergone a significant decline in effective population size, the approximate value of *M* is ≤ 0.70

(Garza & Williamson 2001). I used the parameters suggested by Garza and Williamson for the proportion of single-step mutations (p_s = 90%) and the average size of multi-step mutations (Δ_g = 3.5). As above, I also tested p_s = 78%. The results of the 90% single-step mutation tests for both M-ratio and BOTTLENECK are presented in the Appendix (Table A.4). Garza and Williamson (2001) recommend that sampling approximately 25 diploid individuals is sufficient for most vertebrate populations, although they indicate that there are no clear guidelines for determining an adequate sample size. I tested four critical values of θ ($4N_e\mu$) based on a range of effective population size estimates calculated for bobcat populations in the study area using LDNe as described above. These values corresponded with a N_e of 25 (θ = 0.05), 50 (θ = 0.1), and 150 (θ = 0.3), with a fixed mutation rate (μ) of 5 x 10^{-4} per locus per generation. Statistical significance of the M-ratio was evaluated with 10,000 iterations.

Population structure and genetic differentiation

I investigated the number of genetic clusters and estimated levels of population differentiation geographically and temporally using neutral and immune-linked loci separately. I applied two Bayesian clustering methods that use multilocus genotype data to infer population structure and assign individuals to populations. STRUCTURE 2.3.4 infers population assignments without any *a priori* assumptions about sample location (Pritchard *et al.* 2000). I also used Geneland 4.0.3 that utilized sample locations and genotype data in a spatial statistical model to infer the number of genetic clusters (*K*), as well as the spatial distribution of, and genetic discontinuities between, assigned populations (Guillot *et al.* 2005).

Using STRUCTURE, I first inferred the number of genetic clusters (*K*) without any *a priori* assumptions about the sample location. I performed numerous trials with a minimum of 3

independent runs and with a burn-in period of 50,000 iterations followed by 500,000 MCMC cycles each to evaluate the behavior of the program. The stability of the inferred clusters was then evaluated using 10 independent runs at K = 1-10 with a burn-in period of 50,000 iterations folloId by 500,000 MCMC cycles. Next, for the highest likelihood K value, I evaluated the cluster assignment results for each individual with respect to its capture location, performing analyses with prior information as previously described (Riley *et al.* 2006), and I report these results (Fig. 1.2a-c, Fig. A.1a-c, Table A.5, Appendix). I examined both raw probability values of LnP(K) and the ΔK estimate (Evanno *et al.* 2005) using STRUCTURE HARVESTER (Earl & vonHoldt 2011) to help identify the most likely number of genetic clusters given geographic and temporal data. I used CLUMPP 1.1 (Jakobsson & Rosenberg 2007) to align the multiple STRUCTURE replicates.

STRUCTURE was also used to examine how disease affected population turnover in the mange-affected population. As a result of ongoing NPS radio-telemetry studies in the N101 area (Riley *et al.* 2003; 2010; NPS unplubl. data), yearly survival data for all radio-collared individuals was available. Using these survival data, I grouped individuals into two-year increments, corresponding with the 2-year generation time estimated for bobcats (Knick *et al.* 1985). Each time increment included all animals that were known to be alive during that time period. If individuals were alive for more than two years, they were represented in each time increment they were known to be alive. Using this modified dataset, I performed STRUCTURE analyses as described above.

Finally, I used STRUCTURE to perform assignment tests to identify potential migrants within the dataset. Genetic assignment tests can identify the population of origin for each individual, and individuals that are assigned to a population different than the one in which they

were captured are considered potential migrants (e.g. Berry *et al.* 2004). I modified the STRUCTURE input data file to reassign individuals to their putative population of genetic origin based on the cluster assignment results (see also Riley *et al.* 2006). Briefly, I calculated the posterior probability of correct population assignment with the revised data set using the ancestry model with admixture, incorporating population information with the migration parameter set to v = 0.1. Individuals were considered migrants if their assignment probabilities and posterior probabilities were greater than 50% to a population different than the one in which they were captured. Majority-rule assignments were thus used to identify migrants, and to determine population or origin. This approach may be misleading about the source population of migrants if individuals derive from unsampled populations. However, previous studies showed through radio-telemetry or mark-recapture observations that this procedure often correctly deduces the population of origin if adjoining populations are sampled (Berry *et al.* 2004; Riley *et al.* 2006).

I used Geneland (4.0.3; Guillot *et al.* 2005) to examine spatial and temporal patterns of genetic discontinuities across the landscape. Because genotypes used by the program are georeferenced, I created two data sets to measure the potential temporal difference in population structure across samples collected over the long-term. In addition to examining potential anthropogenic features that were related to genetic discontinuities, I was also interested in whether the disease outbreak influenced genetic discontinuities. Therefore, I assessed two datasets that included: 1) all bobcats sampled from 2006-2012 (E405, S101, and N101post-mange [Fig.1.1]); and 2) bobcats sampled from E405 and S101 during 2006-2012 and bobcats sampled in N101 pre-mange (1996-2001). In both datasets, I also included 10 individuals from the Moorpark satellite population (MRPK, Fig. 1.1) sampled from 2010-2011 to determine which N101 population they assigned to. For each dataset, I ran 500,000 iterations with a

thinning of 100 and K = 1-10. This test was done 5 times with each dataset to evaluate the stability of the most likely K. Migrants could influence genetic discontinuities along the landscape as inferred by Geneland. Therefore, I tested the above datasets with 1) all individuals; and 2) without the observed 4 migrants identified by STRUCTURE (see results).

Finally, to examine the impact of disease and urban development on genetic differentiation, I estimated pairwise F_{ST} using FSTAT 2.9.3.2 (Goudet 1995). The use of F_{ST}, however, has been recently criticized (Jost 2008; Heller & Siegismund 2009), and so I also calculated an alternative estimator of population differentiation, D_{est} (Jost 2008) using the program SMOGD (Crawford 2010). D_{est} more accurately accounts for differences in allelic diversity than F_{ST} particularly for highly polymorphic microsatellite markers (Heller & Siegismund 2009). D_{est} was calculated with 1,000 bootstraps for each marker class. To test the significance of overall D_{est} values for neutral and immune-linked loci across all populations, and the per immune locus values in the N101 populations impacted by disease, I computed probabilities using 999 permutations in GenAlex 6.5 (Peakall & Smouse 2012). For comparison to previous publications, I provide between population F_{ST} values in the Appendix (Table A.6).

Relatedness

I calculated individual pairwise relatedness (R) using the program Maximum-likelihood (ML)-Relate (Kalinowski *et al*. 2006) for each population using neutral loci. I calculated the mean relatedness per population and compared the mean relatedness across populations using an ANOVA, and between population pairs using *t*-tests.

Tests for Selection

I evaluated the role of selection in shaping immune-linked loci variation. I used per locus D_{est} for each of the total 16 neutral and immune-linked microsatellite loci and calculated pairwise comparisons between the two classes of markers for each population pair using Mann-Whitney tests. By comparing the level of divergence derived from the neutral and immune classes of markers, it may be possible to detect whether selection (balancing or diversifying) is acting on immune-linked loci, or whether drift dominates the observed patterns of variation (Piertney & Oliver 2005).

Additionally, for each population pair, I evaluated the deviation from neutrality for all immune and neutral loci using Beaumont and Nichol's Fdist2 method implemented in LOSITAN (Beaumont & Nichols 1996; Antao *et al.* 2008). This approach evaluates the relationship between F_{ST} and expected heterozygosity under an island model of migration with neutral markers and can potentially identify loci under positive or balancing selection. Outlier loci that have excessively high or low F_{ST} compared to neutral expectations are considered potentially under selection. For each population pair, I carried out 100,000 simulations using composite neutral and immune genotypes, assuming a stepwise mutation model.

RESULTS

Bobcat sampling

I obtained 287 blood and tissue samples from live-captured bobcats from four putative bobcat populations (E405, S101, N101 before/during mange and N101 post-mange) from the period 1996-2012. Opportunistic samples were collected postmortem from 39 mortalities from 1996-2012 and 39 scats were collected from 2008-2012. A total of 365 samples (live-trapped,

mortalities, and scat) were used for genotyping at 9 neutral loci. Given limitations on my budget, I was only able to genotype 299 individuals using the seven immune-linked loci that were live-trapped or sampled postmortem (Table A.1, Appendix).

Microsatellite characterization and validation

Of 365 samples genotyped at 9 neutral loci, 3% had data missing for 1 locus, and 1 scat had data missing for 2 loci. Of 299 samples genotyped using 7 immune-linked loci, 3% had data missing for 1 locus, and no samples Ire missing data for more than 1 locus (Table A.7, Appendix). Five neutral and 6 immune-linked loci showed evidence of null alleles, although no loci exhibited evidence of null alleles across all four populations, and thus the evidence of null alleles were likely due to population structure (Tables A.8-A.9, Appendix). No genotyping errors or allele dropout were observed.

Forty-three out of 480 pairs of neutral and immune-linked loci demonstrated significant linkage disequilibrium (LD) after correction for multiple tests. None of the 144 neutral pairs of loci were in significant LD across all populations, and thus the observed significant LD values were likely due to population structure. Eleven of 140 pairs of immune-linked loci showed LD, with three MHC class II gene pairs (DRB3 and DRB1; DRB1 and DRA1; DRB4 and DRB1) in LD across the four bobcat populations. DRB and DRA Class II genes are found within 18.3 Mb of each other on chromosome B2 and have related functions. Specifically, DRB1 and DRB3 are separated by 24 kb, DRB1 and DRB4 are separated by 79 kb, and DRB1 and DRA1 are separated by 18.3 Mb. Consequently, because the linkage of these loci may have influenced the results of our analyses, I performed the tests with and without DRB1, and found no apparent change in the results or significance tests (Tables A.5, A.10-A.11, Fig A.1.b, Appendix).

Seven neutral and three immune-linked loci showed significant deviation from Hardy-Weinberg equilibrium, although none deviated from equilibrium across all four populations (Table A.12, Appendix). Significant heterozygote deficiencies were observed for immune markers including FLA1, DRB1, DRB3 and TLR3 and neutral markers FCA026, FCA090, and FCA086, although not consistently across all four populations (Table A.13, Appendix). Three immune-linked loci (DRB1, DRB3, and TLR3) were observed to be heterozygote deficient in N101 before/during mange, but were not deficient in N101 post-mange, potentially as a result of balancing selection acting on those loci. Heterozygote excess was not observed for neutral or immune-linked markers for any population. For immune-linked markers, a lack of heterozygote excess suggests that ongoing strong selection favoring heterozygotes does not contribute materially to maintaining variation at these loci.

Genetic diversity

All loci were polymorphic with 6-15 alleles observed per neutral locus and 3-10 alleles observed per immune-linked locus. Observed heterozygosity (H_O) ranged from 0.55 to 0.72 for neutral loci, and 0.60 to 0.65 for immune-linked loci (Table 1.2; Table A.14, Appendix). Expected heterozygosity (H_E) ranged from 0.64 to 0.73 for neutral loci and from 0.63 to 0.71 for immune-linked loci. Mean per locus allelic richness (A_R) ranged from 5.22 to 6.88 for neutral markers, and 4.71 to 6.21 for immune-linked markers (Table 1.2; Table A.15, Appendix). Allelic richness ($\chi^2 = 6.93$, $P_{neutral} = 0.07$; $F_{3,13} = 0.97$, $P_{immune} = 0.44$), observed heterozygosity ($F_{3,17} = 2.60$, $P_{neutral} = 0.09$; $\chi^2 = 1.43$, $P_{immune} = 0.70$), and expected heterozygosity ($\chi^2 = 7.08$, $P_{neutral} = 0.07$; $F_{3,13} = 0.94$, $P_{immune} = 0.45$) did not significantly vary across the four populations for either class of marker. These measures also did not significantly vary for immune-linked loci when

DRB1 was excluded (H_O: $F_{3,11}$ = 1.04, P = 0.41; H_E: $F_{3,11}$ = 0.43, P = 0.73; A_R: χ^2 = 4.59, $P_{neutral}$ = 0.20). However, values for each diversity measure and class of marker Were lowest in the most isolated, fragmented eastern population of bobcats (E405), likely as a result of reduced gene flow across the I-405 and complete isolation of the population from source populations other than S101 (Fig. 1.1). For pairwise comparisons between the N101 before mange (1996-2001) and post-mange (2006-2012) populations (Table A.3, Appendix), H_O was significantly lower after the mange outbreak for neutral loci (t_{16} = 2.33, P = 0.03), though not for immune-linked loci. Although H_E and A_R did not differ significantly after the disease outbreak for neutral or immune-linked loci, neutral H_E and A_R Were lower post-disease epizootic (Tables 1.2 and A3, Appendix). A loss of ≥1 rare allele occurred after mange for all neutral loci with the exception of FCA043.

The inbreeding coefficient, F_{IS}, differed significantly across the four populations for neutral loci (χ^2 = 14.92, P = 0.005), although not for immune-linked loci when tested using all 7 loci ($F_{3,15}$ = 2.05, P = 0.15) and when DRB1 was excluded ($F_{3,13}$ = 1.15, P = 0.37) (Tables 1.2 and A.10, Appendix). However, the range of F_{IS} values for both classes of loci was similar (neutral F_{IS} = 0.01-0.14; immune F_{IS} = 0.02-0.14), and thus the lack of significant difference across the four populations for immune-linked loci may be due to insufficient poIr or Type II error. Neutral loci F_{IS} values Were significantly different from zero for E405, S101, and N101 post-mange (P = 0.001 for each population), while immune F_{IS} values Were significantly greater than zero for S101 and N101 before/during mange (P = 0.002 for each population). Neutral F_{IS} pairwise comparisons between E405 and N101 before/during mange, and N101 before/during mange and post-mange, differed significantly (t_{12} = 2.82, P = 0.02 and t_{17} = 2.83, P = 0.01). The E405 neutral inbreeding coefficient (F_{IS} = 0.14; SE = 0.05) was the highest of the four populations and was fourteen times greater than N101 before/during mange (F_{IS} = 0.01; SE =

0.02), the population with the lowest F_{IS}. The inbreeding coefficient measured with neutral loci increased ten-fold for the N101 population post-disease (t_{17} = -2.83, P = 0.01) (Table 1.2) compared with before the disease outbreak.

Relatedness (Table 1.2) was significantly different across the four populations ($F_{3,131}$ = 3.21, P < 0.001). Within population relatedness was highest for N101 post-mange (R = 0.09; SE = 0.003) and E405 (R = 0.09; SE = 0.004) while lowest for S101 (R = 0.08; SE = 0.001) and N101 before/during mange (R = 0.08; SE = 0.001). In pairwise comparisons, E405 was significantly higher than S101 (t_{2694} = 3.57, P < 0.001) and N101 before/during mange (t_{2785} = 3.83, P < 0.001). S101 and N101 before/during mange had significantly lower R compared with N101 post-mange (t_{2273} = -3.99, P < 0.001 and t_{2333} = 4.22, P < 0.001). Relatedness did not significantly differ between E405 and N101 post-mange (t_{4075} = -0.6435, P = 0.52) or S101 and N101 before/during mange (t_{32040} = 0.67, P = 0.50). The significant results reflect the high number of individual pairwise relatedness observations but the relatedness values are very similar. Thus, the biological significance of the differences is questionable. However, the higher relatedness value for E405 may be a consequence of population isolation while the increase in relatedness in N101 post-mange is consistent with a population decline as a result of the disease outbreak.

Demography

For neutral markers, using the program BOTTLENECK, I detected significant heterozygote excess in the N101 population from 2004-2005 (P = 0.007) and 2006-2008 (P = 0.02) using both 78 and 90% single-step mutation parameters, evidence that a bottleneck occurred as a result of the disease outbreak (Tables 1.3 and A.4, Appendix). I also detected a

deviation from the expected L-shaped allele distribution for neutral loci in 2004-2005 indicating that a significant shift in allele frequencies occurred as a result of the population decline. I did not detect heterozygote excess for any other population.

I additionally tested for population bottlenecks using Garza and Williamson's *M*-ratio test. *M*-ratios ranged from 0.67 to 0.89 for neutral loci across all populations and years (Tables 1.3 and A.4, Appendix). I detected a bottleneck in the N101 population during years 2004-2005 (M = 0.69), 2006-2008 (M = 0.71) and 2009-2012 (M = 0.67).

The effective population size, N_e, for bobcats found north of the 101 Freeway (N101) before mange was 47 (95% CI: 25.9-130.7) individuals during 1996-1998 and 17.6 (95% CI: 12.1-27.6) individuals during 2000-2001. During the final years of the mange outbreak, N_e was 9 (95% CI: 5.6-13) individuals, the lowest value across all sample year increments (Table 1.1). Although the effective population size estimates have overlapping confidence intervals across most years for the N101 population, the decline in the effective population size during the mange outbreak is consistent with field observations of fewer bobcats in N101 as a result of the disease outbreak. For bobcats east of the I-405 (E405), the effective population size was estimated to be 34.4 (95% CI: 21.8-61.2) between years 2010-2011. Combining all bobcats captured south of the101 Freeway (S101) from 1996-2012, N_e was estimated to be 97.7 (95% CI: 71-143.6).

Population assignments and genetic differentiation

I used the Evanno method (Evanno *et al.* 2005) to infer the optimal number of clusters in STRUCTURE. Two (K = 2) population clusters had the highest posterior probability assignments for neutral loci (Table A.5, Appendix). However, population clusters resolved at K = 4 corresponded better with our geographic and temporal knowledge of bobcat substructure (Fig.

1.2a). In fact, Evanno *et al.* (2005) caution that Δ*K* may be used to help identify the correct number of clusters in most situations but that it should not be used exclusively. Partial sampling of individuals in a population has been shown to lead to a lower Δ*K* than the true value, and for a cryptic species such as the bobcat, complete population sampling would not be feasible. Evanno *et al.* (2005) suggest that Δ*K* should be considered in conjunction with other information provided by STRUCTURE such as individual assignment patterns. Moreover, Geneland resolved population assignments concordant with the four genetic clusters determined in STRUCTURE (Fig. 1.2d-g). Geographically, populations from both STRUCTURE and Geneland included individuals captured east of I-405 (E405), south of the 101 Freeway (S101), and two clusters north of the 101 Freeway (N101) divided temporally. The first N101 population included individuals sampled from 1996-2005 (STRUCTURE) or 1996-2001 (Geneland) in the mange-affected region, along with the Moorpark satellite group of individuals (N = 10) sampled northwest of the mange-affected region in 2010-2011. The second population north of the 101 Freeway included all animals sampled post epizootic (2006-2012), with the exception of the Moorpark individuals that clustered with N101 before/during mange population. Based on assignment probabilities and posterior probabilities of greater than 50% (Table A.16, Appendix), only one bobcat captured in N101 genetically assigned with the S101 population, while 3 bobcats captured in S101 genetically assigned to E405.

When STRUCTURE was used to examine population turnover in N101 based on survival of individuals over two-year increments, two clusters ($K = 2$) were resolved (Fig. 1.2b, Table A.5, Appendix). A clear, progressive pattern of genetic turnover is present suggesting that the population was reestablished by a small group of founders that originated from the N101 population.

Both freeways and disease influenced population differentiation (Tables 1.4 and A.6 and A.11, Appendix). Using neutral markers, all pairwise comparisons of D_{est} (Table 1.4) showed genetic differentiation between populations (D_{est} = 0.06 - 0.12) with a global D_{est} = 0.10. All probability calculations for neutral D_{est} were significant at $P \leq 0.001$. The greatest D_{est} values were observed between the E405 and N101 populations that are geographically separated by two major freeways. For the N101 before/during mange and N101 post-mange populations D_{est} = 0. 08, a value greater than for those populations separated by a major freeway (Table 1.4).

Using immune markers, all pairwise comparisons of D_{est} (Table 1.4) also showed genetic differentiation between populations (D_{est} = 0.02 - 0.17) with a global D_{est} = 0.09. All probability calculations for immune D_{est} were significant at $P \leq 0.002$. Using immune markers, overall genetic differentiation between populations was lowest between N101 before/during mange and post-mange (D_{est} = 0.02) and highest between E405 and N101 post-mange (D_{est} = 0.17).

Tests of selection

Given the significant D_{est} values, I was surprised that using STRUCTURE and Geneland, no clear population structure was resolved when tested using immune-linked loci (Fig. 1.2c, Fig. A.1, Appendix), though our F_{ST} values are considered borderline for resolving population structure using STRUCTURE (Pritchard *et al.* 2000). However, population subdivision should be less for markers under balancing selection because of the greater effective migration rate of alleles (Scheierup *et al.* 2000). Therefore, these findings suggest that immune-linked loci may under balancing selection. Alternatively, the absence of structure for the immune-linked loci may represent lower power when using 7 immune-linked loci vs. 9 neutral loci. In pairwise D_{est} comparisons of the two classes of markers, a significant difference between neutral and immune-

linked loci was found only between N101 before/during mange and post-mange (W = 10.5, P = 0.03) (Table 1.4). This finding was consistent when tested without DRB1 (W = 8.5, P = 0.03) (Table A.11, Appendix). Thus, I can only conservatively conclude that strong balancing selection across multiple immune-linked loci has likely acted only on the N101 before/during and post-disease populations. Nonetheless, the absence of population structure for immune-linked loci is suggestive of balancing selection. Consequently, to evaluate the degree to which fewer immune-linked loci may influence the resolution of population structure, I performed an additional STRUCTURE analysis with 6 neutral loci chosen based on their similarities to the immune-linked loci with respect to their number of alleles and allele frequency distributions. I found that less structure was resolved, although more structure than for immune-linked loci when tested using 6 loci (Fig. A.1c, Appendix). Thus, in conclusion, differences in the number of alleles (Table A.17, Appendix) or allele frequency spectra between the 2 classes of markers may influence the resolution of population structure. In addition, differential patterns of per locus selection and drift across the different bobcat populations may also have contributed to the observed absence of structure for immune-linked loci when tested using STRUCTURE (Fig. 1.2h).

Measures of per immune-linked locus differentiation for the N101 before/during and post-disease population pair revealed TLR3, TLR4 and FLA1 to each have a D_{est} = 0.00 (Table 1.5). For loci DRA1 and DRB1, D_{est} values were lower than overall neutral values (D_{est} = 0.02 and 0.06), while DRB3 and DRB4 had D_{est} values similar to the overall neutral value (D_{est} = 0.08 and 0.10). Using probability tests, none of the per-locus D_{est} values were significant (Table 1.5) between N101 before/during mange and N101 post-mange. Although no outlier loci were

detected using LOSITAN, the program authors report that the program is ill-equipped to detect low F_{ST} outliers as F_{ST} approaches zero (Antao *et al.* 2008).

Inbreeding coefficients showed evidence of both balancing selection and genetic drift acting on immune-linked loci in SMMNRA. The S101 population had the highest inbreeding coefficient at immune-linked loci, greater than for neutral loci and equivalent with the E405 neutral inbreeding coefficient. Considering differences in isolation, population size and historical demography, the similarity of immune inbreeding coefficients in S101 and E405 is puzzling. In contrast, the E405 population had the greatest inbreeding coefficient using neutral loci (Table 1.2) and yet a significantly lower inbreeding coefficient measured using immune-linked loci (t_{16} = -2.26, P = 0.04), suggesting the influence of balancing selection on immune-linked loci in this population. Further, although F_{IS} measured using neutral loci increased 10-fold in N101 post-disease, immune-linked loci F_{IS} decreased by more than half. The decrease was not significant when comparing N101-before/during mange and post-mange (t_{13} = -1.57, P = 0.14), but when N101 before and post-mange were compared (Table A.3, Appendix), the difference was significant (W = 13, P = 0.05). Per-locus decreases in F_{IS} were also observed for multiple immune-linked loci when comparing N101 before/during mange with post-mange (Table 1.5). TLR3, FLA1, DRB1, and DRB3 differed significantly from zero in N101 before/during disease (P = 0.005, 0.03, 0.005 and 0.02), but decreased in N101 post-mange and no longer differed significantly from zero. DRB4, in contrast, increased significantly from 0.06 to 0.17, suggesting inbreeding in the small population during the disease outbreak.

DISCUSSION

Population structure

Urban development is increasingly recognized to have important genetic impacts on wildlife populations (Munshi-South & Kharchenko 2010; Delaney *et al*. 2010), especially for large, highly mobile species such as bobcats (Riley *et al*. 2006; 2010; Lee *et al*. 2012). I evaluated the roles that freeways and disease had on genetic structure and differentiation in urban bobcat populations and identified four population groupings that clustered temporally and spatially. Populations separated by freeways or before and after a disease outbreak were the most differentiated. Neutral measures of genetic diversity were lowest in the most isolated, fragmented population (E405) as well as in the N101 post-mange population, a population that suffered a population bottleneck caused by a disease outbreak. These findings indicate the freeways and disease are important contributors to genetic diversity and differentiation in bobcat populations in SMMNRA.

Roads and freeways are a common feature of most landscapes and may impose an important barrier to movement fragmenting animal populations (Forman & Alexander 1998). Within SMMNRA, many secondary roads are present, although they were not observed to contribute to population structure. Both major freeways in the study area were important barriers to movement and gene flow. Specifically, the I-405 was a greater barrier to gene flow than the 101 Freeway. Further, genetic movement across the I-405 appears unidirectional moving east to west perhaps due to the limited available habitat east of the I-405. Higher population differentiation across the I-405 may also be the result of fewer potential wildlife crossing points; the I-405 has only four potential points whereas the 101 Freeway has seven. The influence of the 101 Freeway on population structure and genetic differentiation is consistent with previous

results (Riley *et al.* 2006) although the number of migrants differed. Riley *et al*. (2006) detected 13 individuals genetically assigned to the opposite side of the freeway from where they were captured. However, our dataset includes a greater number of individuals and loci, and so I potentially had more power to detect true migrants. Nonetheless, I detected only 4 migrants, one of which crossed the 101 Freeway from S101 to N101 post-mange, and 3 of which crossed from E405 to S101. Because I sampled approximately twice as many individuals in E405 as in the area directly west of the I-405, I do not believe my migrant detection for the areas straddling the I-405 to be biased by my sampling design.

In southern California and especially within my study area, a variety of species have been documented to cross freeways through culverts and passageways under freeways (Ng 2004). Riley *et al*. (2006) found through radio-telemetry observations that four bobcats crossed the 101 Freeway. Although no radio-telemetry studies have been performed near the I-405, 26 remote wildlife cameras were recently placed at four I-405 crossing points and adjacent habitat for 19 months. Although bobcats were detected 247 times by cameras in habitat adjacent to crossing points, they were detected only twice by cameras in passageways suggesting limited bobcat movement across the freeway (Schoonmaker & Riley 2011). Interestingly, nine samples from Griffith Park (GP) assign with the E405 population although the park is isolated from this population by the 101 Freeway (Fig. 1.1). Thus, east of the I-405, the 101 Freeway may pose less of a barrier to movement than in other regions of SMMNRA. In general, however, the E405 population is perhaps the most threatened by roads and isolation in the Los Angeles area. Habitat in the E405 region, consisting largely of the Hollywood Hills, is the most fragmented in SMMNRA and has no direct connection to other populations except S101. Although I did not detect a genetic bottleneck in the E405 population, the genetic diversity measures, including for

immune-linked loci, were lowest in the E405 population, lower even than for the bottlenecked N101 post-mange population. Population isolation and decreased genetic diversity may cause this population to be more vulnerable to other stressors associated with urbanization and increase susceptibility to disease. In fact, this population is presently experiencing a decline associated with a notoedric mange outbreak (Serieys, unpubl.data) further threatening its long-term stability. Within SMMNRA, there are no wildlife corridors across freeways, and although underpasses may be used (Ng 2004), my results highlight the need for improved connectivity across increasingly urbanized and fragmented landscapes to prevent population isolation of bobcats and possibly other low to moderate abundance species such as mountain lions (*Puma concolor*, Beier *et al.* 2010), gray foxes (*Urocyon cinereoargenteus,* Temple *et al.* 2010), grizzly bears (*Ursus arctos,* Gibeau *et al.* 2002), among others (Ng 2004). The design and implementation of such corridors across large roadways must become an essential feature of developing megacities where the preservation of rare native species at the urban-wildlife interface is a priority.

My results also indicated that disease can be as strong an influence on population structure as freeways. Genetic differentiation between the N101 before/during mange and the N101 post-mange populations was greater than for populations separated by freeways. The disease outbreak caused a decline of > 50% on the survival rate of bobcats radio-collared by NPS, and uncollared animals were also found dead with the disease (Riley *et al.* 2007; 2010). The population appears to be recovering (Riley, personal communication) and based on the survivor STRUCTURE analysis, a small group of founders likely originating in N101 were the source of the founder population.

In a metapopulation, local turnover due to stochastic factors such as disease can increase genetic differentiation among local populations. However, the mode in which new populations

are founded will influence the degree of population differentiation (Wade & McCauley 1988). When the number of colonizers is large and individuals originate from multiple source populations, turnover will have a homogenizing effect decreasing differentiation (Slatkin 1977). Alternatively, if the number of colonizers is small and originate from only a few source populations, genetic drift is accelerated by the small number of founders and turnover can increase population differentiation (Harrison & Hastings 1996; Pannell & Charlesworth 2000). The degree of differentiation between the N101 before/during and post- disease populations and S101 suggests a small number of N101 individuals survived to establish the post-mange population, rather than having been repopulated by individuals from nearby populations (e.g. S101 or Moorpark).

Genetic effects of a population bottleneck

I found evidence that the N101 bobcat population underwent a bottleneck coincident with the notoedric mange outbreak from 2002-2005. Because a bottleneck signature was observed only in N101, the bottleneck was likely not a phenomenon that occurred across the region. The strong genetic evidence of a population bottleneck is consistent with field observations made by NPS biologists that have been radio-collaring and tracking bobcats in the area since 1996 (Riley *et al.* 2007; 2010). These data illustrate the value of coupling genetic and field studies, particularly for secretive wide-ranging carnivores such as bobcats.

With large reductions in effective population size, genetic changes from random genetic drift include a shift in allele frequencies, a loss of rare alleles, and a decrease in heterozygosity (Nei *et al*. 1975; Kimura 1985; Dlugosch & Parker 2008). From 1996-1998, the N101 population had a high effective population size of 47 individuals, which dropped to a low of approximately

9 individuals from 2004-2005 towards the end of the notoedric mange outbreak. Consistent with the decrease in effective population size to a small number of founding individuals, I detected a significant increase in inbreeding and relatedness in the post-disease N101 population based on neutral loci. However, I observed only a slight nonsignificant decrease in allelic richness and observed heterozygosity after the disease epizootic. The strength of a bottleneck and the rate of population recovery affects average heterozygosity and the number of alleles per locus (Nei *et al.* 1975). If population recovery occurs quickly or the bottleneck is small, significant decreases in heterozygosity or allelic richness may not be detected (Nei *et al.* 1975). Because bobcats are notoriously elusive and difficult to census by direct observation, the rate at which the population recovered is unclear. However, based on capture rates and reproduction observations, the population began recovery after approximately 7 years, or roughly 3 bobcat generations (Knick *et al.* 1985) after the beginning of the mange epizootic (Riley *et al.* NPS unpubl. data).

Evidence of selection

Population declines can lead to genetic consequences that reduce individual fitness and viability and compromise adaptive potential (Frankham *et al.* 1999). Both genetic diversity in neutral and immune-linked loci can be lost as a result of genetic drift during population bottlenecks (Sutton *et al.* 2011). However, strong selection at functional loci may counteract genetic drift and maintain adaptively important genetic variation (Aguilar *et al.* 2004; Piertney & Oliver 2005; Oliver & Piertney 2012), even in populations that have undergone extreme genetic bottlenecks (Aguilar *et al.* 2004; Piertney & Oliver 2005; Whittaker *et al.* 2012). To my knowledge, I am the first to evaluate how genetic drift and selection influence genetic variation at immunologically important loci in an urban free-ranging wildlife population. Further, the

majority of immunogenetic studies have focused on MHC, while very few have examined patterns of genetic diversity at toll-like receptor loci, particularly in a free-ranging populations.

I observed a significant discrepancy in the degree of genetic differentiation at immune and neutral loci between N101 before/during mange and N101 post-mange. For immune-linked loci, genetic differentiation was absent for TLR3, TLR4 and FLA1, while DRA1 had less than half the differentiation observed at neutral markers. Among my most striking results was the significant decrease in immune-linked loci F_{IS} simultaneous to a significant, 10-fold increase in F_{IS} for neutral markers as a result of the disease outbreak. A low inbreeding coefficient at functional genetic regions in comparison with the inbreeding coefficient measured at neutral loci suggests balancing selection (Black *et al.* 2001; Oliver & Piertney 2012). Specifically, I observed a decrease in F_{IS} values for FLA1, DRB1, DRB3, and TLR3 after the disease outbreak, implying balancing selection acting on these loci. In contrast, I observed a significant increase in the inbreeding coefficient for DRB4. Balancing selection is unlikely to affect all immune-linked loci, particularly given their varied functional roles in the vertebrate immune system. I know of only one study that has found evidence of balancing selection affecting variation at TLR regions (Ferrer-Admetlla *et al.* 2008), whereas my results indicated balancing selection on both TLR loci and 4 of 5 MHC loci as a result of a disease outbreak associated with secondary anticoagulant poison exposure.

Previous studies that have compared the degree of differentiation between neutral markers and immune genes in wildlife populations have reported varying degrees and types of selection to influence divergence in populations (Bernatchez & Landry 2003; Piertney & Oliver 2005; Tschirren *et al.* 2011; Bollmer *et al.* 2011). The lower genetic divergence in immune-linked loci compared with neutral markers observed in remnant populations of Malagasy

jumping rat (*Hypogeomys antimena*, Sommer 2003), greater prarie-chickens (*Tympanuchus cupido*, Bollmer et al. 2011), and island foxes (*Urocyon littoralis*, Aguilar et al. 2004) was attributed to balancing selection. For Eastern Atlantic grey seals (*Halichoerus grypus*) geographic patterns in MHC variation and differentiation Ire consistent with habitat-specific pathogen pressure (Cammen *et al.* 2010).

Relatively few studies have addressed patterns of genetic differentiation in toll-like receptor regions compared with neutral markers. Lower TLR2 differentiation compared with neutral divergence in yellow-necked mouse (*Apodemus flavicollis*) populations was attributed to a selective sweep and 95% of individuals shared the same haplotype (Tschirren *et al.* 2011). On the other hand, greater genetic variation at TLR2 compared with neutral markers observed in bank voles (*Myodes glareolus*) was attributed to diversification and local adaptation (Tschirren *et al.* 2011). Finally, heterogeneity in selection pressure reflected varying degrees of balancing selection detected in toll-like receptor and other markers involved in innate immunity in European human populations compared with African populations (Ferrer-Admetlla *et al.* 2008).

Mange infection in the N101 population and selection on immune related genes

The roles of specific immune genes in response to mange infection remains elusive, though it may involve both innate and adaptive immunity pathways (Arlian *et al.* 2004; Walton *et al.* 2008). I observed both a lack of genetic differentiation between before/during and post-disease populations, and a decrease in F_{IS} for the FLA1 locus, suggesting MHC Class I gene involvement in bobcat immune response to notoedric mange. I also observed a decrease in F_{IS} for DRB1 and DRB3, suggesting that MHC Class II-mediated immune response may have been important for bobcats infested with notoedric mange. The scabies mite, *Sarcoptes scabiei*, is

closely related to notoedric mange mites, and in humans, an immune response dominated by a Th1 cell-mediated protective response is thought to promote parasite control (Walton *et al.* 2008; Walton 2010). The Th1 immune response is dominated by CD4+ (helper T) and CD8+ (cytotoxic T) cells that interact directly with MHC class I and II molecules (Huang & Germain 1992). A recent study found evidence that MHC Class II *DRB*-mediated immune response to scabies (*S.rupicaprae*) may increase survival of male Alpine chamois (*Rupicapra rupicapra*) during rutting season (Schaschl *et al.* 2012). I have documented that bobcats with mange experience leukocytosis, and specifically significantly elevated neutrophil, monocyte and eosinophil counts (Serieys *et al.* 2013). Although neutrophils have key function in innate immune response, they express MHC Class I molecules (Neuman *et al.* 1992) and are able to stimulate adaptive immune response (Potter & Harding 2001).

Toll-like receptors may similarly have been important to the initiation of bobcat immune response to mange infection. The observed decrease in TLR3 F_{IS} and absence of differentiation for TLR3 and TLR4 between N101 before/during mange and N101 post-mange suggests TLR involvement during the disease outbreak. TLR signaling activates antigen presenting cells that support helper and cytotoxic T cell differentiation (Kaisho & Akira 2006). Additionally, monocytes, neutrophils, and eosinophils, each found elevated in bobcats with severe mange (Serieys *et al.* 2013), express TLR4 (Sabroe *et al.* 2002; Iwasaki & Medzhitov 2010). In neutrophils, TLR4 expression plays a critical role in prolonged cell survival (Sabroe *et al.* 2003) while TLR3 activation of eosinophils is thought to enhance eosinophil ability to concentrate at inflamed tissues in humans (Månsson *et al.* 2010). Across SMMNRA, I have tested bobcats for exposure to ten common feline viral and bacterial pathogens and have not detected unusually high prevalence of any disease with the exception of mange (Serieys *et al*. unpubl. data).

Therefore, the observed patterns of selection across immune-linked loci are likely driven primarily by severe and widespread mange parasitism in the region.

Balancing selection in other populations

Differentiation in neutral and immune-linked loci is similar and non-significant across other populations, suggesting the latter are not experiencing divergent natural selection or under balancing selection in other areas of SMMNRA. I also observe an absence of population structure for immune-linked loci that may reflect the limited power of 7 loci, qualitative differences in variation of the neutral and immune markers, as well as differential patterns of drift and selection acting on individual immune-linked loci across the study area. One suggestive finding is that F_{IS} for neutral loci is 7 times greater than for immune-linked loci in the E405 population, a difference similar to that for the N101 post-mange population. Notoedric mange has been observed in E405 since at least 2006, and presently appears to be a primary source of mortality for bobcats in the population (Serieys, unpubl.data). Thus, balancing selection may also be an important regulator of genetic variation at immune-linked loci in the E405 population in response to mange parasitism. Previous studies have generally found that variation at immune-linked loci is correlated with variation at neutral markers, suggesting the influence of genetic drift on immune-linked loci (e.g. Atlantic salmon, *Salmo salar*, Landry & Bernatchez 2001; New Zealand robins, *Petroica traverse*, Miller & Lambert 2004; and bighorn sheep *Ovis canadensis mexicana*, Hedrick & Espeleta 2001). Overall, as in our study, relatively few studies find consistent patterns of selection acting on immune regions across subdivided populations, and the variability is generally attributed to heterogeneity in effective population sizes, challenge by pathogens, and landscape features in subdivided populations (Bernatchez & Landry 2003).

Conservation Implications

Our data highlight the importance of increased urbanization on the genetic structure and diversity of animal populations and suggests that efforts to ameliorate these effects are critical to long-term conservation and effective management. Most surprising is the influence of a disease epizootic on population differentiation in the region affected by mange. I have observed a strong association between bobcat secondary exposure to anticoagulant rodenticides and increased risk of mortality due to severe notoedric mange (Riley *et al.* 2007; Serieys *et al.* unpubl.data), a disease that is now affecting numerous bobcat populations across the State of California (Serieys *et al.* 2013; Stephenson *et al.* 2013). Bottlenecks can reduce genetic variation and thereby threaten the long-term viability of wildlife populations. The Florida panther experienced a severe demographic contraction as a result of human depredation and encroachment that depleted genetic diversity and led to severe fitness costs (Roelke *et al.* 1993). Populations depleted of genetic variation may also become more susceptible to infectious disease (O'Brien & Evermann 1988). Bottlenecked populations of bighorn sheep (*Ovis aries*) are highly susceptible to infectious disease, although their MHC diversity remains high (Espeleta *et al.* 2001). For California sea lions (*Zalophus californianus*), inbred individuals were suggested to be more effective reservoirs for disease than individuals with greater genetic diversity (Acevedo-Whitehouse *et al.* 2003). Although balancing selection may have acted to maintain functionally important genetic variation in the N101 population, our results raise the concern that notoedric mange can have fundamental impacts on the genetics of certain populations, particularly those that are isolated or located at the urban-wildlife interface where stress is greater.

Roads and urban infrastructure may also have profound impacts on genetic variation and differentiation in bobcats. The greater Los Angeles area has a population of approximately 18 million people, is among the megacities of the world (DEMOGRAPHIA), and is expected to experience a population increase of 61.7% by 2050 (www.america2050.org). As urbanization continues in Los Angeles and elsewhere, disease and decreased connectivity may become critical factors in the persistence of many populations. Wildlife corridors across freeways are one critical measure that may assist in the persistence of genetic diversity across rapidly urbanizing landscapes. Additionally, maintaining habitat with minimal edge is important. For example, in my study area, anticoagulant poisoning occurs primarily in areas that are associated with urban development, likely reflecting the placement of rodenticides outside homes and outdoor facilities (Serieys *et al.* unpubl. data). In more core habitat areas in SMMNRA, mange and poison exposure is less frequent (Serieys *et al.* 2013). Thus, connected habitats with natural buffers where disease or toxicant effects have less opportunity to permeate across the wildlife-urban interface and favorable adaptive genetic variation can spread, will be critical for the long-term viability of wildlife populations in urban landscapes.

TABLES AND FIGURES

Table 1.1. Effective population size (Ne) and 95% parametric confidence intervals for bobcat populations in SMMNRA. For the N101 population affected by the notoedric mange epizootic, N_e is partitioned by sampling years.

Population	Year	N	N_e	95% CI
N101	1996-1998	33	47.2	25.9 - 130.7
	2000-2001	22	17.6	12.1 - 27.6
	2002*****	22	14.3	9.9 - 21.8
	2003*****	25	14.6	10.8 - 20.1
	2004-2005*****	22	8.6	5.6 - 13
	2006-2008	20	13.3	8.3 - 22.9
	2009-2012	30	14.5	9.4 – 23.0
S101	1996-2012	126	97.7	71 - 143.6
E405	2010-2011	47	34.4	21.8 - 61.2

***** Years during which the mange epizootic occurred.

Table 1.2. Genetic diversity measures and standard errors for four bobcat populations in SMMNRA. Standard errors are shown in parentheses. Neutral loci measures are shown to the left and immune-linked loci measures are to the right within columns.

Population	H_O	H_E	AR	F_{IS}	R
E405	0.55 (0.06) / 0.60 (0.05)	0.64 (0.04) / 0.63 (0.03)	5.22 (0.57) / 4.71 (0.69)	0.14 (0.05) / 0.02 (0.04)	0.09 (0.004)
S101	0.65 (0.06) / 0.61 (0.05)	0.73 (0.02) / 0.70 (0.04)	6.88 (0.73) / 6.08 (0.92)	0.09 (0.05) / 0.14 (0.05)	0.08 (0.001)
N101-B/DM	0.72 (0.03) / 0.64 (0.06)	0.73 (0.03) / 0.70 (0.06)	6.84 (0.62) / 6.21 (0.80)	0.01 (0.02) / 0.08 (0.03)	0.08 (0.001)
N101-PM	0.61 (0.04) / 0.65 (0.05)	0.68 (0.03) / 0.71 (0.05)	5.93 (0.75) / 6.14 (0.76)	0.10 (0.03) / 0.03 (0.03)	0.09 (0.003)

Table 1.3. Results of bottleneck tests using BOTTLENECK and *M*-ratio using 78% single-step mutations. Values in bold are significant ($P \leq 0.05$) indicators of a genetic bottleneck. Varying θ values correspond with multiple pre-bottleneck effective population size estimates where $\theta = 0.05$ ($N_e = 25$), $\theta = 0.1$ ($N_e = 50$), and $\theta = 0.3$ ($N_e = 150$).

			BOTTLENECK			*M*-ratio *P*		
Population	Year	N	TPM‡	Mode shift	*M*-ratio	$\theta = 0.05$	$\theta = 0.1$	$\theta = 0.3$
N101	1996-1998	34	0.179	no	0.839	0.245	0.282	0.386
	2000-2001	23	0.082	no	0.809	0.145	0.171	0.263
	2002*****	22	0.102	no	0.792	0.111	0.130	0.202
	2003*****	26	0.326	no	0.810	0.156	0.178	0.259
	2004-2005*****	23	**0.002**	**yes**	**0.690**	**0.011**	**0.012**	**0.021**
	2006-2008	21	**0.007**	no	**0.708**	**0.014**	**0.021**	**0.033**
	2009-2012	31	0.367	no	**0.666**	**0.005**	**0.006**	**0.011**
S101	1996-2001†	30	0.125	no	0.894	0.507	0.545	0.671
	2006-2012†	26	0.064	no	0.788	0.105	0.115	0.183
	2008-2012	85	0.150	no	0.806	0.1500	0.162	0.249
E405	2010-2011	48	0.177	no	0.799	0.165	0.193	0.284

***Years during which the mange epizootic occurred.**

† Malibu Creek State Park subset of S101 population only.

‡ TPM: two-phase model

Table 1.4. Jost's D_{est} for 9 neutral and 7 immune-linked loci, and *P*-values for pairwise comparisons of immune and neutral D_{est} estimates for each population pair. All probability calculations for D_{est} values are significant. Value in bold represents a significant difference between the two classes of markers at $P \leq 0.05$.

	D_{est}		
Population pairs	Neutral	Immune	*P*
E405 – S101	0.06	0.09	0.68
E405 – N101-B/DM	0.11	0.11	0.69
E405 – N101-PM	0.12	0.17	0.54
S101 – N101-B/DM	0.05	0.03	0.46
S101 – N101-PM	0.05	0.04	0.38
N101-B/DM – N101-PM	0.08	0.02	**0.03**

Table 1.5. Per immune locus and overall neutral inbreeding coefficient (F_{IS}) and genetic differentiation (D_{est}) values for the N101 populations. Standard error values are in parentheses for composite F_{IS} values. Values in bold are significant at $P \leq 0.05$. Significance was calculated using permutation tests.

		F_{IS}		
Class	Locus	N101-B/DM	N101-PM	D_{est}
Neutral	All neutral (S.E.)	0.01 (0.02)	**0.10** (0.03)	**0.08**
MHC class I	FLA1	**0.10**	0.06	0.00
MHC class II	DRA1	-0.04	0.02	0.02
	DRB1	**0.15**	-0.12	0.06
	DRB3	**0.08**	0.04	0.08
	DRB4	0.06	**0.17**	0.10
Toll-like receptor	TLR3	**0.16**	-0.06	0.00
	TLR4	0.02	0.02	0.00
Immune	All immune (S.E.)	**0.08** (0.03)	0.02 (0.03)	0.03

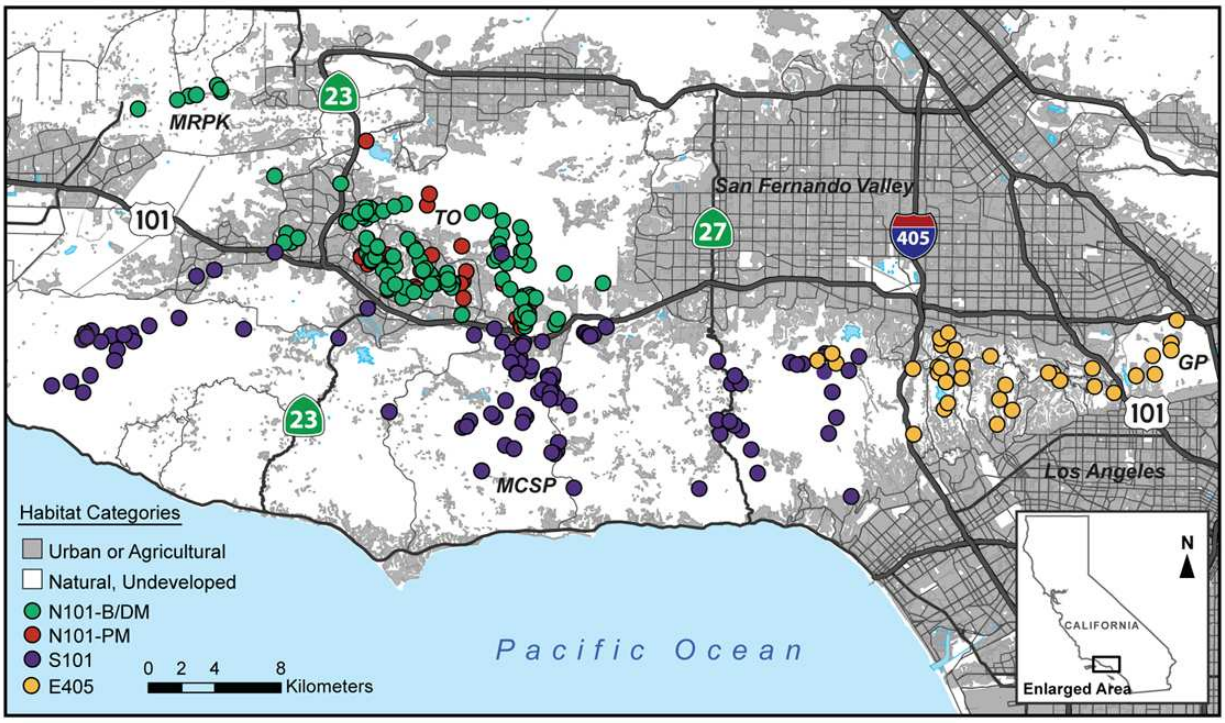
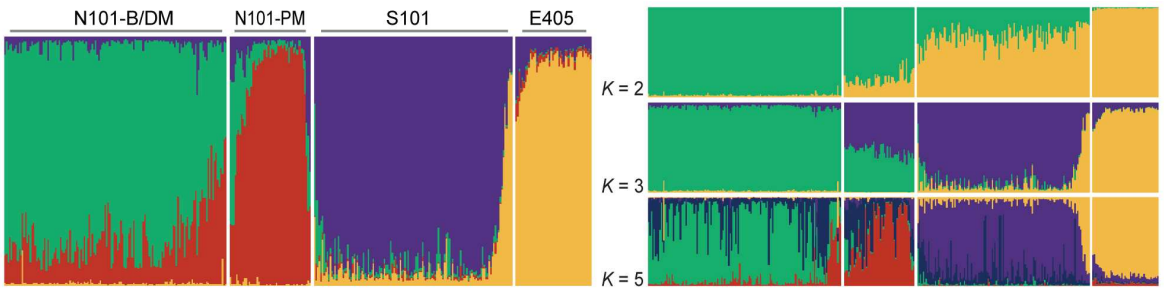
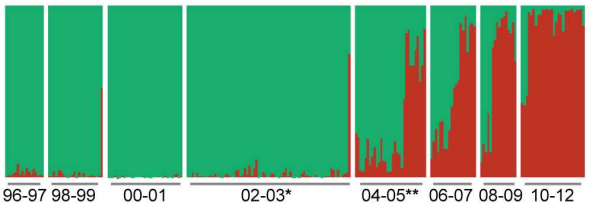


Figure 1.1. Map of Santa Monica Mountains National Recreation Area where bobcats were sampled. Colored circles represent individual bobcat capture locations and colors correspond with the predominant STRUCTURE cluster assignment for each individual. Major freeways of interest include the 101 Freeway and I-405. The 23 (south of the 101 Freeway) and the 27 are secondary roads that intersect the study area. Specific locations referenced in the study: MRPK (Moorpark satellite population), TO (Thousand Oaks), MCSP (Malibu Creek State Park), and GP (Griffith Park). N101-B/DM: N101 before/during mange; N101-PM: N101 post-mange.

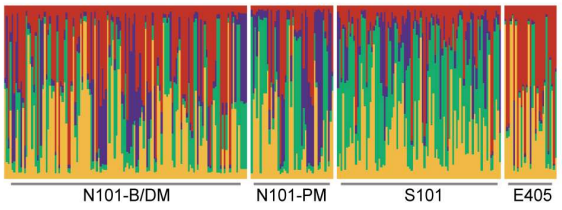
Figure 1.2. Results of population structure analyses, using capture location as prior information, using neutral loci (a-b, d-g) and immune-linked loci (c). For STRUCTURE plots (a-c), each vertical bar represents one individual. The shading of each bar corresponds to the probability of genetic assignment to one of four populations of bobcats that included N101 before/during mange (N101-B/DM, green), N101 post-mange (N101-PM, red), S101 (purple), or E405 (gold). (a) $K = 4$ results for bobcats across the study area. (**b**) Analysis ($K = 2$) using survival information for individuals in the N101 population from 1996-2012. * indicates when mange entered the population, ** indicates when a genetic bottleneck is detected. (**c**) $K = 4$ results for bobcats across the study area using immune-linked loci. No distinguishable structure was present for any *K*-value. (d-g) Geneland results, interpolated over SMMNRA, based on runs with migrants removed. Black circles represent bobcat sampling locations. Color assignments correspond with STRUCTURE results. Darker colors represent a high probability of assignment to a focal population, while the lighter color represents a high probability of assignment to any other population. (**d**) N101 before/during mange resolved (green). (**e**) N101 post-mange resolved (red). (**f**) S101 resolved (purple). (**g**) E405 resolved (gold). Admixture between N101 and S101 resulted in unclear population assignment boundaries in the westernmost region of SMMNRA in (d) and (f). (**h**) Per locus D_{est} values for all population pairs. A significant difference between the overall neutral and immune D_{est} values was present only for the N101-B/DM – N101-PM population pair.



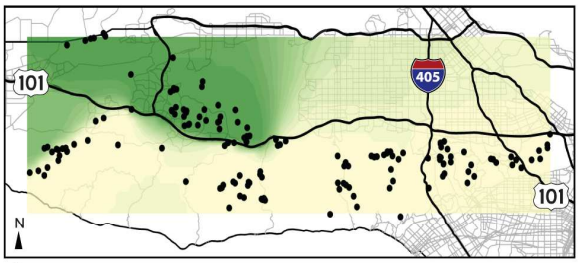
a *K* = 4 (left) and *K* = 2, 3 and 5 (right) using 9 neutral loci.



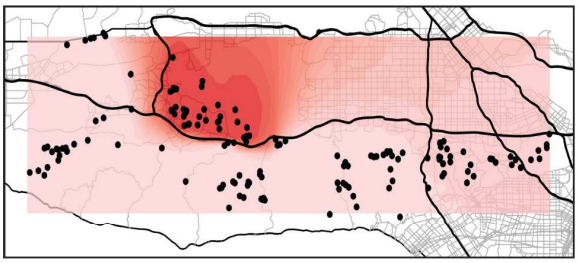
b Survivor analysis (*K* = 2) for bobcats in N101.



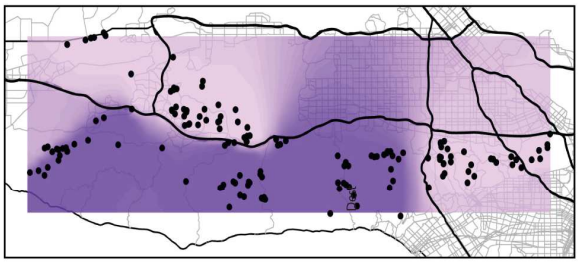
c *K* = 4 using immune loci.



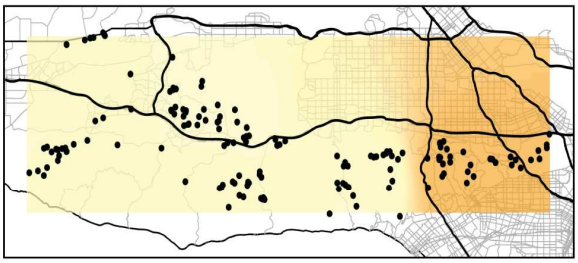
d N101-B/DM resolved in Geneland.



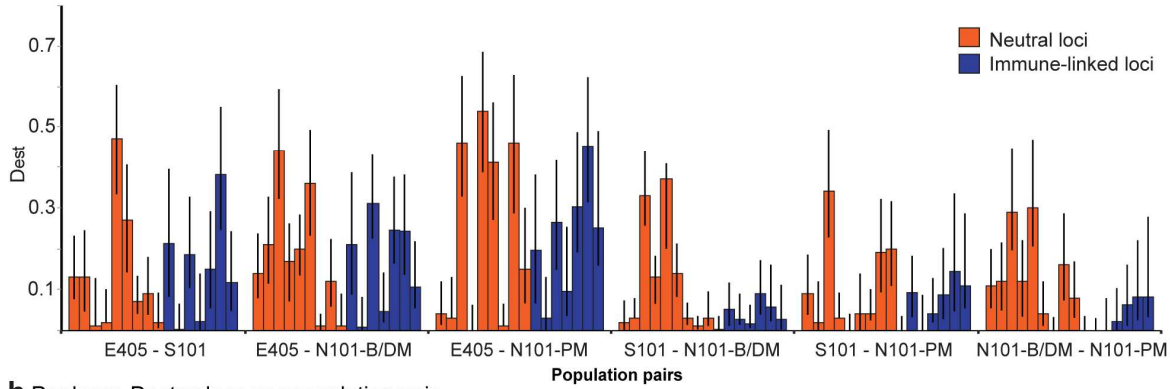
e N101-PM resolved in Geneland.



f S101 resolved in Geneland.



g E405 resolved in Geneland.



h Per locus Dest values per population pair.

APPENDIX

Table A.1. The number of individuals (N) and proportion of each bobcat sample type per population used for genotyping. The values to the left of the backslash represent proportions of each sample type for genotyping using neutral loci, while the values to the right are for immune-linked loci.

Population	N	Antemortem tissue	Postmortem tissue	Buccal swab	Scat
E405	49 / 29	0.56 / 0.91	0.09 / 0.03	0.00 / 0.00	0.35 / 0.00
S101	124 / 91	0.59 / 0.82	0.22 / 0.12	0.05 / 0.06	0.14 / 0.00
N101-B/DM	141 / 135	0.94 / 0.98	0.04 / 0.01	0.01 / 0.01	0.01 / 0.00
N101-PM	51 / 44	0.72 / 0.84	0.06 / 0.00	0.14 / 0.16	0.08 / 0.00

Table A.2. Information for immune-linked microsatellite loci and primers developed for this study.

Locus	Primer (5′–3′)	Length (kb)	Repeat	Relation to target region
FLA1	F: ACACACTGAGCACCAAGCAC	189	CA	20 kb downstream of FLA1
	R: CCCTGCTCACACTCTGTCTG			
TLR3	F: CCCCTCCAGTTCTGCAATAA	267	TG	8 kb downstream from TLR3
	R: GCGAGACTGTAGGCAGTTCC			
TLR4	F: GCTTCTCCCTAAATGCTGCC	251	TGGA	Intron of TLR4
	R: ACCTCAATGGACTGCCCTC			
DRA1	F: CCCGTGCCTGTTATCAACTT	216	GA	2 kb upstream from DRA1
	R: GGGTATGATGCCTTCTCCAA			
DRB1	F: GCCCTGATGAGGTCAGCC	287	TTTA	5 kb upstream from DRB1
	R: GATAGAGTCCCAGGTCGGG			
DRB3	F: TCTCACGTTTTGTGGGTGTG	248	CT	14 kb upstream from DRB3
	R: TGAATCCTTCTTGCGGAACT			
DRB4	F: ATGGCTCCCAAGGCAAAGG	273	CA	Intron of DRB4
	R: CAAGAGTTGCATGCCCTACC			

Table A.3. Genetic diversity measures for the N101 population stratified by mange status: before, during, and after the mange epizootic. Values calculated with 9 neutral loci are on the left, and 7 immune-linked loci are to the right of the backslash.

Mange status	Years	N	H_O	H_E	AR	F_{IS}
Before	1996-2001	56 / 53	0.73 / 0.65	0.73 / 0.71	7.02 / 6.63	-0.01 / 0.08
During	2002-2005	71 / 70	0.71 / 0.62	0.73 / 0.68	6.19 / 6.32	0.02 / 0.09
After	2006-2012	51 / 46	0.61 / 0.65	0.68 / 0.71	5.99 / 6.14	0.10 / 0.03

Table A.4. **Results of bottleneck tests using BOTTLENECK and *M*-ratio using 90% single-step mutations. Values in bold are significant ($P \leq 0.05$) indicators of a genetic bottleneck. Varying θ values correspond with multiple pre-bottleneck effective population size estimates where $\theta = 0.05$ ($N_e = 25$), $\theta = 0.1$ ($N_e = 50$), and $\theta = 0.3$ ($N_e = 150$).**

			BOTTLENECK			*M*-ratio *P*		
Population	Year	N	TPM‡	Mode shift	*M*-ratio	$\theta = 0.05$	$\theta = 0.1$	$\theta = 0.3$
N101	1996-1998	34	0.455	no	0.839	0.04	0.051	0.082
	2000-2001	23	0.213	no	0.809	0.017	0.024	0.04
	2002*****	22	0.150	no	0.792	0.009	0.015	0.026
	2003*****	26	0.455	no	0.810	0.021	0.023	0.043
	2004-2005*****	23	**0.007**	**yes**	**0.690**	**0.001**	**0.000**	**0.001**
	2006-2008	21	**0.019**	no	**0.708**	**0.000**	**0.001**	**0.002**
	2009-2012	31	0.545	no	**0.666**	**0.000**	**0.000**	**0.000**
S101	1996-2001†	30	0.326	no	0.894	0.162	0.186	0.273
	2006-2012†	26	0.180	no	0.788	0.011	0.011	0.023
	2008-2012	85	0.248	no	0.806	0.016	0.020	0.037
E405	2010-2011	48	0.411	no	0.799	0.012	0.017	0.03

***Years during which the mange epizootic occurred.**

† Malibu Creek State Park subset of S101 population only.

‡ TPM: two-phase model

Table A.5. The harvested results of STRUCTURE analyses. Using the Evanno method, K = 2 was the most optimal number of clusters for each analysis shown.

Analysis	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln"(K)|	Delta K
9 neutral loci, all bobcats	1	10	-10222.14	0.2	–	–	–
	2	10	-9965.61	5.96	256.53	144.44	24.25
	3	10	-9853.52	11.27	112.09	25.63	2.27
	4	10	-9715.8	15.69	137.72	13.65	0.87
	5	10	-9591.73	14.58	124.07	64.45	4.42
	6	10	-9532.11	16.47	59.62	23.71	1.44
	7	10	-9496.2	70.86	35.91	7.66	0.11
	8	10	-9452.63	48.97	43.57	35.39	0.72
	9	10	-9444.45	55.24	8.18	66.86	1.21
	10	10	-9503.13	121.77	-58.68	–	–
9 neutral loci, N101 2-year survivor	1	10	-6890.65	0.21	–	–	–
	2	10	-6648.92	1.57	241.73	46.38	29.48
	3	10	-6453.57	13.07	195.35	50.54	3.87
	4	10	-6308.76	9.02	144.81	44.59	4.95
	5	10	-6208.54	36.55	100.22	12.16	0.33
	6	10	-6096.16	16.9	112.38	104.47	6.18
	7	10	-6088.25	155.34	7.91	148.55	0.96
	8	10	-5931.79	29.09	156.46	120.96	4.16
	9	10	-5896.29	38.2	35.5	14.29	0.37
	10	10	-5875.08	58.58	21.21	–	–
7 immune-linked loci, all bobcats	1	10	-10222.14	0.2	–	–	–
	2	10	-9965.61	5.96	256.53	144.44	24.25
	3	10	-9853.52	11.27	112.09	25.63	2.27
	4	10	-9715.8	15.69	137.72	13.65	0.87
	5	10	-9591.73	14.58	124.07	64.45	4.42
	6	10	-9532.11	16.47	59.62	23.71	1.44
	7	10	-9496.2	70.86	35.91	7.66	0.11
	8	10	-9452.63	48.97	43.57	35.39	0.72
	9	10	-9444.45	55.24	8.18	66.86	1.21
	10	10	-9503.13	121.77	-58.68	–	–
6 loci immune-linked loci (DRB1 excluded), all bobcats	1	10	-5452.85	0.14	–	–	–
	2	10	-5183.59	0.9	269.26	67.67	75.19
	3	10	-4982	5.48	201.59	113.03	20.62
	4	10	-4893.44	41.27	88.56	38.32	0.93
	5	10	-4766.56	46.5	126.88	22.69	0.49
	6	10	-4662.37	8.43	104.19	31.63	3.75
	7	10	-4589.81	12.72	72.56	25.2	1.98
	8	10	-4542.45	30.7	47.36	76.06	2.48
	9	10	-4571.15	95.4	-28.7	94.26	0.99
	10	10	-4694.11	240.33	-122.96	–	–

Table A.6. ***F*ST values for each population pair calculated using 9 neutral loci and 7 immune-linked loci.**

	F_{ST}	
Population pairs	Neutral	Immune
E405 – S101	0.065	0.062
E405 – N101-B/DM	0.067	0.068
E405 – N101-PM	0.102	0.094
S101 – N101-B/DM	0.035	0.017
S101 – N101-PM	0.035	0.026
N101-B/DM – N101-PM	0.048	0.010

Table A.7. The proportion of missing data for each locus per population. The sample type and number of samples for which data are missing are specified in parentheses.

Locus	E405	S101	N101-B/DM	N101-PM
FCA008	0.00	0.02 (N = 3, scat)	0.00	0.00
FCA023	0.00	0.01 (N = 1, scat)	0.00	0.00
FCA026	0.00	0.00	0.00	0.00
FCA043	0.00	0.01 (N = 1, antemortem tissue)	0.00	0.00
FCA045	0.00	0.00	0.00	0.00
FCA077	0.00	0.01 (N = 1, postmortem tissue)	0.00	0.00
FCA090	0.02 (N = 1, scat)	0.00	0.00	0.02 (N = 1, scat)
FCA096	0.00	0.02 (N = 2, buccal swab; N = 1, scat)	0.00	0.00
FCA132	0.00	0.01 (N = 1, scat)	0.00	0.02 (N = 1, scat)
FLA1	0.00	0.01 (N = 1, post-mortem tissue)	0.02 (N = 3, antemortem tissue)	0.00
DRA1	0.00	0.00	0.00	0.00
DRB1	0.00	0.00	0.01 (N = 1, antemortem tissue)	0.00
DRB3	0.00	0.01 (N = 1, post-mortem tissue)	0.00	0.00
DRB4	0.00	0.02 (N = 1, postmortem tissue; N = 1, antemortem tissue)	0.00	0.00
TLR3	0.00	0.00	0.01 (N = 1, antemortem tissue)	0.00
TLR4	0.00	0.00	0.00	0.00

Table A.8. Neutral locus null allele frequency estimates per population using 4 methods implemented in Micro-checker.

Population	Locus	Evidence of null alleles?	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
E405	FCA008	no	-0.02	-0.02	-0.02	0.00
	FCA023	no	0.04	0.03	0.02	0.02
	FCA026	no	0.02	0.02	0.02	0.02
	FCA043	no	0.09	0.11	0.07	0.07
	FCA045	yes	0.12	0.14	0.08	0.08
	FCA077	no	0.08	0.10	0.07	0.07
	FCA090	yes	0.17	0.24	0.11	0.18
	FCA096	yes	0.16	0.21	0.13	0.13
	FCA132	no	-0.02	-0.02	-0.01	0.00
S101	FCA008	no	-0.01	-0.01	0.00	0.07
	FCA023	no	0.01	0.01	0.01	0.05
	FCA026	no	0.00	0.00	0.00	0.00
	FCA043	no	0.01	0.02	0.01	0.05
	FCA045	yes	0.23	0.33	0.19	0.19
	FCA077	no	0.04	0.05	0.04	0.07
	FCA090	no	0.04	0.03	0.03	0.03
	FCA096	yes	0.07	0.07	0.05	0.11
	FCA132	no	0.00	0.00	0.00	0.06
N101-B/DM	FCA008	no	0.00	0.00	0.00	0.00
	FCA023	no	-0.05	-0.05	-0.04	0.00
	FCA026	yes	0.07	0.07	0.06	0.06
	FCA043	no	0.03	0.03	0.03	0.03
	FCA045	no	0.00	0.00	0.00	0.00
	FCA077	no	-0.03	-0.03	-0.02	0.00
	FCA090	no	0.02	0.03	0.02	0.02
	FCA096	no	0.03	0.02	0.02	0.02
	FCA132	no	-0.02	-0.01	-0.01	0.00
N101-PM	FCA008	no	0.03	0.03	0.02	0.02
	FCA023	no	0.05	0.05	0.04	0.04
	FCA026	no	0.03	0.04	0.03	0.03
	FCA043	no	0.04	0.04	0.03	0.03
	FCA045	yes	0.08	0.10	0.07	0.07
	FCA077	no	-0.03	-0.03	-0.02	0.00
	FCA090	yes	0.13	0.15	0.09	0.19
	FCA096	no	0.05	0.05	0.04	0.04
	FCA132	no	0.07	0.06	0.05	0.11

Table A.9. Immune-linked locus null allele frequency estimates per population using 4 methods implemented in Micro-checker.

Population	Locus	Evidence of null alleles?	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
E405	FLA1	no	0.11	0.11	0.07	0.07
	DRB3	no	0.00	0.02	0.02	0.02
	DRB1	no	-0.05	-0.05	-0.04	0.00
	DRA1	no	-0.01	-0.01	-0.01	0.00
	TLR3	no	-0.01	0.00	0.00	0.00
	TLR4	no	0.04	0.04	0.03	0.03
	DRB4	no	-0.05	-0.04	-0.04	0.00
S101	FLA1	yes	0.19	0.23	0.16	0.18
	DRB3	yes	0.09	0.10	0.08	0.11
	DRB1	no	0.04	0.05	0.04	0.04
	DRA1	no	-0.02	-0.02	-0.01	0.00
	TLR3	no	0.04	0.04	0.03	0.03
	TLR4	yes	0.11	0.13	0.07	0.07
	DRB4	no	0.02	0.02	0.02	0.08
N101-B/DM	FLA1	no	0.05	0.05	0.04	0.12
	DRB3	yes	0.04	0.04	0.03	0.03
	DRB1	yes	0.08	0.08	0.06	0.09
	DRA1	no	-0.03	-0.03	-0.02	0.00
	TLR3	yes	0.07	0.08	0.06	0.09
	TLR4	no	0.01	0.01	0.00	0.00
	DRB4	no	0.03	0.03	0.02	0.02
N101-PM	FLA1	no	0.03	0.03	0.03	0.03
	DRB3	no	0.02	0.02	0.02	0.08
	DRB1	no	-0.09	-0.07	-0.06	0.00
	DRA1	no	0.02	0.01	0.01	0.01
	TLR3	no	-0.04	-0.03	-0.02	0.00
	TLR4	no	0.01	0.02	0.01	0.01
	DRB4	yes	0.09	0.10	0.08	0.08

Table A.10. Genetic diversity measures calculated using immune-linked loci for each bobcat population. The values for the total 7 immune-linked loci are to the left of the backslash, and the values calculated using 6 loci (DRB1 excluded) are to the right**.**

Statistic	E405	S101	N101-B/DM	N101-PM
Allelic richness	4.57 / 4.17	6.25 / 6.02	6.21 / 5.92	6.23 / 5.90
F_{IS}	0.02 / 0.04	0.14 / 0.15	0.08 / 0.07	0.03 / 0.05
Observed heterozygosity	0.60 / 0.58	0.61 / 0.61	0.64 / 0.65	0.65 / 0.65
Expected heterozygosity	0.63 / 0.62	0.70 / 0.71	0.70 / 0.70	0.71 / 0.71

Table A.11. Jost's D_{est} for each population pair for 9 neutral and 6 immune-linked loci (DRB1 excluded), and *P*-values for pairwise comparisons of immune and neutral D_{est} estimates of genetic differentiation for each population pair. Value in bold is significant at $P \leq 0.05$.

	D_{est}		
Population pairs	Neutral	Immune	*P*
E405 – S101	0.06	0.08	0.86
E405 – N101-B/DM	0.11	0.09	0.69
E405 – N101-PM	0.12	0.15	0.61
S101 – N101-B/DM	0.05	0.02	0.38
S101 – N101-PM	0.05	0.03	0.36
N101-B/DM – N101-PM	0.08	0.01	**0.03**

Table A.12. Per locus Hardy-Weinberg probability values for each population.

Locus type	Locus	E405	S101	N101-B/DM	N101-PM	Across all populations
Neutral	FCA008	0.735	0.126	0.609	0.225	0.004
	FCA023	0.084	0.297	0.401	0.023	0.001
	FCA026	0.938	0.000	0.000	0.124	0.000
	FCA043	0.316	0.516	0.375	0.091	0.000
	FCA045	0.546	0.000	0.401	0.439	0.000
	FCA077	0.030	0.485	0.001	0.358	0.000
	FCA090	0.000	0.581	0.024	0.031	0.013
	FCA096	0.076	0.151	0.046	0.163	0.000
	FCA132	0.134	0.039	0.099	0.009	0.000
Immune	FLA1	0.049	0.000	0.611	0.198	0.000
	DRA1	0.162	0.411	0.501	0.347	0.000
	DRB1	0.874	0.151	0.026	0.254	0.017
	DRB3	0.051	0.058	0.066	0.357	0.137
	DRB4	0.396	0.043	0.055	0.001	0.062
	TLR3	0.865	0.753	0.013	0.306	0.000
	TLR4	0.687	0.033	0.455	0.669	0.081

Table A.13. Per locus heterozygosity excess (EXC) and deficiency (DEF) probability values for each population and across all populations. Values in bold are significant at $P \leq 0.05$ after correction for 32 test statistical tests per population ($\alpha = 0.002$).

Locus type		**All**		**E405**		**S101**		**N101-B/DM**		**N101-PM**	
	Locus	EXC	DEF	EXC	DEF	EXC	DEF	EXC	DEF	EXC	DEF
Neutral	FCA008	0.297	0.703	0.371	0.654	0.387	0.615	0.851	0.158	0.012	0.988
	FCA023	0.583	0.417	0.847	0.155	0.638	0.362	0.987	0.013	0.036	0.964
	FCA026	1.000	**0.000**	0.809	0.192	0.259	0.741	0.847	0.156	0.995	0.005
	FCA043	0.912	0.088	0.97	0.031	0.879	0.122	0.651	0.353	0.372	0.628
	FCA045	1.000	**0.000**	0.992	0.01	1.000	**0.000**	0.999	**0.001**	0.059	0.941
	FCA077	0.926	0.074	0.968	0.033	0.965	0.035	0.318	0.688	0.018	0.982
	FCA090	1.000	**0.000**	1.000	**0.000**	0.875	0.126	0.961	0.04	0.989	0.011
	FCA096	1.000	**0.000**	1.000	**0.000**	0.973	0.027	0.841	0.167	0.13	0.871
	FCA132	0.911	0.089	0.855	0.145	0.736	0.264	0.733	0.268	0.434	0.566
Immune	FLA1	1.000	**0.000**	0.963	0.039	1.000	**0.001**	0.978	0.022	0.988	0.012
	DRA1	0.950	0.05	0.593	0.554	0.536	0.465	0.179	0.821	0.469	0.536
	DRB1	0.999	**0.001**	0.351	0.676	0.988	0.013	0.998	**0.002**	0.112	0.907
	DRB3	1.000	**0.000**	0.995	0.005	0.997	0.003	0.999	**0.001**	0.816	0.184
	DRB4	0.997	0.004	0.431	0.605	0.764	0.237	0.813	0.187	0.988	0.012
	TLR3	0.993	0.007	0.55	0.464	0.867	0.135	0.999	**0.001**	0.365	0.639
	TLR4	0.939	0.061	0.806	0.376	0.993	0.023	0.738	0.294	0.652	0.448

Table A.14. Per locus observed and expected heterozygosities per population. Expected heterozygosities are in parentheses.

Locus type	Locus	E405	S101	N101-B/DM	N101-PM
Neutral	FCA008	0.70 (0.67)	0.76 (0.78)	0.75 (0.75)	0.62 (0.66)
	FCA023	0.58 (0.64)	0.69 (0.74)	0.88 (0.78)	0.62 (0.69)
	FCA026	0.82 (0.85)	0.89 (0.85)	0.70 (0.80)	0.75 (0.81)
	FCA043	0.52 (0.62)	0.65 (0.67)	0.69 (0.74)	0.64 (0.69)
	FCA045	0.40 (0.53)	0.29 (0.61)	0.76 (0.76)	0.52 (0.64)
	FCA077	0.52 (0.63)	0.64 (0.76)	0.81 (0.77)	0.77 (0.74)
	FCA090	0.29 (0.45)	0.60 (0.68)	0.52 (0.55)	0.39 (0.54)
	FCA096	0.40 (0.63)	0.56 (0.67)	0.64 (0.68)	0.62 (0.69)
	FCA132	0.74 (0.72)	0.80 (0.78)	0.77 (0.73)	0.57 (0.65)
Immune	FLA1	0.45 (0.61)	0.47 (0.74)	0.67 (0.75)	0.65 (0.75)
	DRA1	0.65 (0.65)	0.73 (0.72)	0.79 (0.76)	0.78 (0.74)
	DRB1	0.74 (0.71)	0.60 (0.66)	0.61 (0.72)	0.67 (0.72)
	DRB3	0.58 (0.65)	0.67 (0.83)	0.78 (0.85)	0.76 (0.85)
	DRB4	0.65 (0.63)	0.76 (0.80)	0.74 (0.80)	0.72 (0.80)
	TLR3	0.71 (0.70)	0.61 (0.66)	0.53 (0.63)	0.60 (0.67)
	TLR4	0.42 (0.47)	0.39 (0.49)	0.38 (0.39)	0.41 (0.44)

Table A.15. Allelic richness per locus per bobcat population.

Locus type	Locus	E405	S101	N101-B/DM	N101-PM
Neutral	FCA008	5.00	6.52	4.90	5.97
	FCA023	4.00	4.91	4.90	5.86
	FCA026	9.00	12.11	10.70	12.45
	FCA043	4.00	4.94	4.99	4.99
	FCA045	3.00	6.84	6.00	7.30
	FCA077	5.96	6.18	6.00	6.57
	FCA090	5.00	5.85	4.92	5.06
	FCA096	5.00	6.75	6.00	6.86
	FCA132	5.98	7.84	4.92	6.51
Immune	FLA1	4.00	6.52	6.37	7.63
	DRB3	3.00	4.00	4.00	4.00
	DRB1	7.00	7.67	7.99	8.23
	DRA1	4.00	9.15	8.02	7.17
	TLR3	6.00	7.22	6.67	5.99
	TLR4	2.00	2.00	2.63	2.99
	DRB4	6.00	7.21	7.82	7.62

Table A.16. STRUCTURE cluster assignment and posterior probability of correct genetic population assignment of potential migrants in SMMNRA.

Animal ID	Direction	Structure Analysis Result: Capture Location Cluster ((%) – Cluster Assignment)	Structure Analysis Result: Cluster Location Assignment (Genetic Origin) ((%) – Cluster Assignment)	Posterior Probability of Correct Assignment to Genetic Origin Cluster
BM016	S101 -> N101-PM	0.31	0.54	0.71
B207	E405 -> S101	0.14	0.85	0.99
B272	E405 -> S101	0.27	0.72	0.75
BS25	E405 -> S101	0.17	0.82	0.97

Table A.17. The number of alleles per locus per population. Subtle qualitative differences, such as in the number of alleles for immune-linked loci compared with neutral loci, may contribute to the absence of STRUCTURE observed in the immune-linked loci STRUCTURE analysis.

	Neutral loci									Immune-linked loci						
Populations	FCA 008	FCA 023	FCA 026	FCA 043	FCA 045	FCA 077	FCA 090	FCA 096	FCA 132	FLA 1	DRA 1	DRB 1	DRB 3	DRB 4	TLR 3	TLR 4
All bobcats	8	6	15	5	8	6	6	8	8	8	12	4	9	8	9	3
E405	5	4	9	4	3	7	5	5	6	5	7	4	9	5	4	2
S101	8	5	14	5	7	6	6	7	8	7	11	4	6	8	9	2
N101-B/DM	5	5	11	5	6	7	5	6	5	7	12	4	8	8	8	3
N101-PM	7	6	15	5	8	7	6	8	7	8	7	4	8	7	6	3

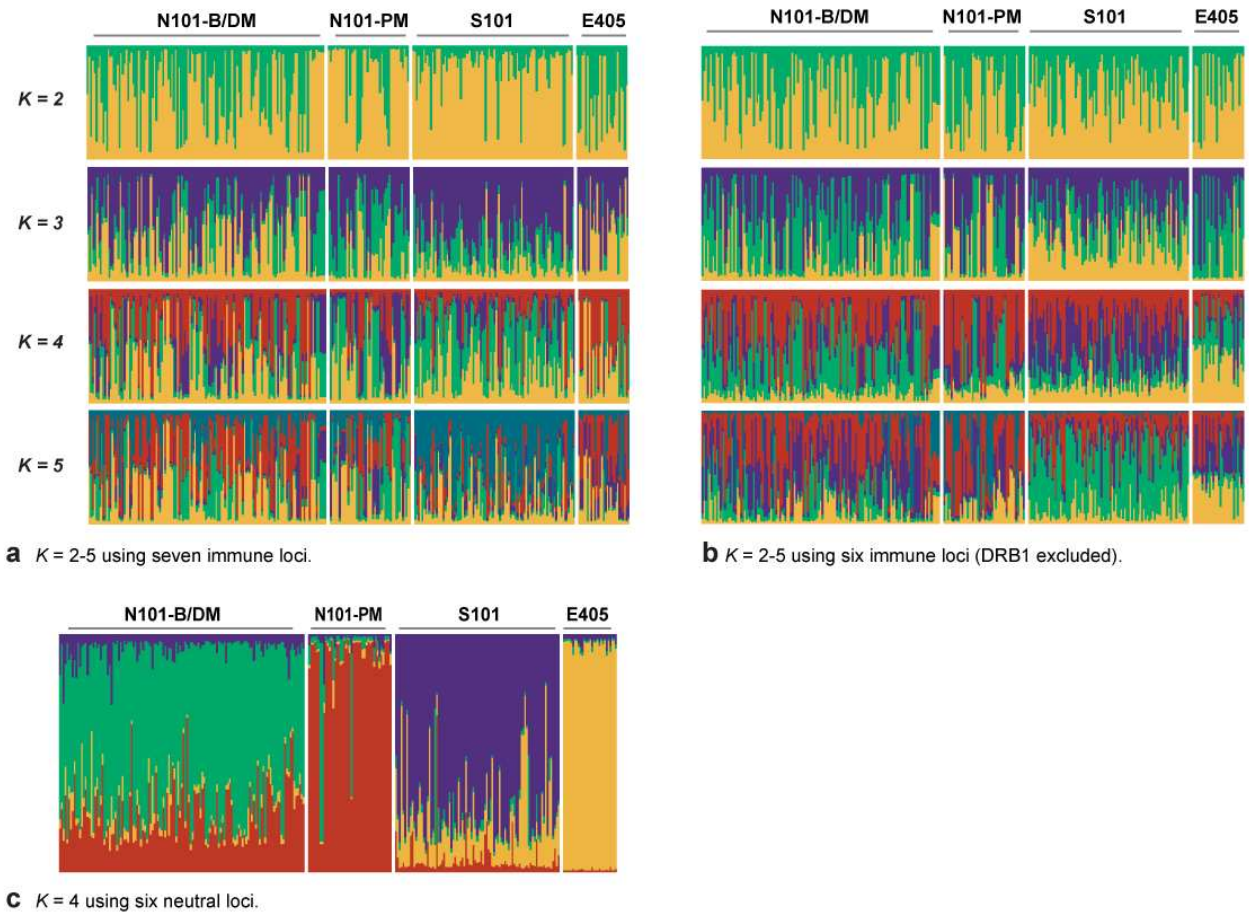


Figure A.1. Results of population structure analyses using immune-linked and neutral loci. N101-B/DM: N101 before/during mange population; N101-PM: N101 post-mange population. (**a**) Analysis performed with 7 immune-linked loci for *K* = 2-5. (**b**) Analysis performed with 6 immune-linked loci for *K* = 2-5. DRB1, found to be in linkage disequilibrium with 3 other immune-linked loci, was excluded from the analysis. (**c**) Analysis performed with 6 neutral loci. Less population structure was resolved in comparison with 9 neutral loci, although still more structure than resolved with 6 (b) or 7 immune-linked loci (a).

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CHAPTER 2

Anticoagulant rodenticides in urban bobcats:

measuring exposure, risk factors, and consequences

ABSTRACT

Toxicants are a leading cause of population decline for a variety of animal species worldwide. One group of compounds, anticoagulant rodenticides (ARs), is increasingly recognized as a threat to nontarget wildlife. High exposure prevalence to ARs and associated mortalities have been documented in nontarget predatory species globally, including in southern California, where AR exposure has also been linked to high prevalence of an ectoparasitic disease, notoedric mange, which has been the proximate cause of a population decline of bobcats (*Lynx rufus*). Over a 16-year period (1997-2012), I examined AR exposure in bobcats across southern California, contrasting seasonal, demographic and spatial risk factors in both natural and urbanized areas. I used a novel approach by sampling both blood (N = 206) and liver (N = 172) to examine exposure ante- and post-mortem. I detected high prevalence of exposure (89%, liver; 39%, blood) and found that for individuals with paired liver and blood data (N = 64), 92% were exposed most frequently to ≥ 3 compounds. Prevalence and the amounts of contaminants were associated with human activities that included commercial, residential, and agricultural development. In particular, AR exposure was strongly associated with proximity to single-family high-density residential communities and golf courses. During the dry season, animals were 2.6 times more likely to be exposed to ARs. Bobcats of both sexes and all age classes were at high risk of exposure, and I documented fetal transfer of multiple ARs. I found a strong association between AR exposure to ≥ 0.25 ppm or ≥ 2 compounds and an ectoparasitic disease, notoedric mange. Results show AR exposure is extremely prevalent throughout the regions sampled, and over the course of the 16-year study. ARs may pose a substantial threat to bobcats, and likely other species living at the urban-wildland interface in southern California.

INTRODUCTION

Anthropogenic development can have profound ecological impacts. Consequences may be obvious, such as habitat loss and fragmentation, or cryptic, such as the exposure of wildlife to toxicants (Fleischli et al. 2004, Kobayashi et al. 2005, Riley et al. 2007, Brar et al. 2010). Exposure to chemical contaminants is considered the third leading cause of species endangerment in the United States (Wilcove and Master 2005). The indiscriminate use of chemical pesticides used to target plant or animal pests may bioaccumulate (Mendelssohn and Paz 1977, Riley et al. 2007, Gehrt and Riley 2010, Alonso et al. 2012), leading to widespread primary or secondary exposure of nontarget species with unintended consequences. Consequences of exposure can include direct mortality (Mendelssohn and Paz 1977, Aguilar and Borrell 1994, Riley et al. 2003, Gehrt and Riley 2010, Gabriel et al. 2012), or sublethal, chronic effects such as reproductive impairment (McMurry et al. 1995, Vos et al. 2000, Eeva et al. 2003, Choi et al. 2004, Scheuhammer et al. 2007), decreased immune competence (Choi et al. 2004), and increased disease susceptibility or emergence (Bradley and Altizer 2007, Riley et al. 2007).

Anticoagulant rodenticides (ARs) represent a group of toxicants increasingly recognized for the threat they pose to nontarget wildlife (Erickson and Urban 2004, US EPA 2008, Elmeros et al. 2011, Gabriel et al. 2012, California Department of Pesticide Regulation 2013). As Vitamin K antagonists, ARs interrupt the production of Vitamin K-dependent blood clotting proteins, leading to the depletion of these proteins over a period of days, and induce mortality by hemorrhage (Erickson and Urban 2004). Comprised of two classes of compounds, they are the primary chemical method of rodent control used worldwide for the control of rats and mice (Stone et al. 1999, Eason et al. 2002). First-generation anticoagulant rodenticides (FGARs), including warfarin, diphacinone, and chlorophacinone, are readily metabolized, have a short

half-life in hepatic tissue ranging from 2 weeks to several months (Eason et al. 2002), and must be consumed in multiple feedings to reach a lethal dose (Erickson and Urban 2004). In contrast, second-generation anticoagulant rodenticides (SGARs) that include brodifacoum, bromadiolone, and difethialone, were developed to target rats with genetic resistance to warfarin (Hadler and Buckle 1992). They have prolonged action and increased potency (Petterino and Paolo 2001), and with hepatic half-lives ranging 6-12 months, persist in liver tissue for more than one year in some species (Eason et al. 2002). Both classes of compounds have delayed onset of action, and death from AR consumption can occur up to 10 days after ingestion (Cox and Smith 1992). Individual rodents may continue to accumulate the compounds over a period of days, increasing their attractiveness to predators as they become weakened by the toxicant, and become easier to capture (Cox and Smith 1992, Berny et al. 1997, Berny 2007). For predatory species that consume prey species targeted with ARs, there is evidence that both acute and chronic secondary exposure to the toxicants can occur (Erickson and Urban 2004, Riley et al. 2007, Elmeros et al. 2011, Gabriel et al. 2012).

The exposure of nontarget wildlife to ARs has been documented for numerous predatory mammal and bird species globally (McDonald et al. 1998, Stone et al. 1999, Riley et al. 2003, 2007, McMillin et al. 2008, Walker et al. 2008, Elmeros et al. 2011). When prevalence of AR exposure is examined in wildlife, detection rates can be high, exceeding 80-90%, and ARs are responsible for direct mortalities in many populations including coyotes (*Canis latrans*; Riley:2003vv), San Joaquin kit foxes (*Vulpes macrotis mutica;* McMillin:2008vg), California fishers (*Martes pennant;*Gabriel:2012bi), mountain lions (*Puma concolor*; Riley et al. 2007), red kites (*Milvus milvus;* Berny and Gaillet 2008), barn owls (*Tyto alba*), barred owls (*Strix varia*) and great horned owls (*Bubo virginianus*) (Albert et al. 2009), among many others. The factors

that lead to secondary exposure of nontarget species are complex (Eason et al. 2002, Shore et al. 2006, Laakso et al. 2010) because exposure is related to the persistence of compounds, levels of usage, how and where the compounds are used, and trophic ecology (Eason et al. 2002, Shore 2003, Erickson and Urban 2004, Shore et al. 2006). The study of AR exposure in wildlife is also difficult because it often relies on post-mortem sampling of liver tissue from carcasses found opportunistically. This may lead to a bias towards detection of those compounds with the longest persistence in hepatic tissue, and an underestimation of the number of animals that are exposed to ARs, and that die directly, or indirectly, of AR consumption.

In southern California, more than a decade of research in and around Santa Monica Mountains National Recreation Area (SMMNRA), a national park bordering the Los Angeles area, has documented widespread AR exposure in multiple carnivore species. AR exposure was the second leading cause of mortality during a 9-year coyote study in which 83% of individuals tested were exposed (Riley et al. 2003, Gehrt and Riley 2010). Ninety percent of mountain lions (*Puma concolor*) and bobcats in the study area were also exposed (Riley et al. 2007, 2010, Beier et al. 2010, NPS unpubl. data). AR toxicant load, or the concentration of AR residues detected, was positively associated with the use of developed areas by radio-collared bobcats and mountain lions (Riley et al. 2007, Beier et al. 2010) suggesting that urban areas are a major source of AR contamination.

Although high rates of exposure were documented for bobcats in SMMNRA, death by anticoagulant toxicity has been reported in only one case (Riley et al. 2010). However, secondary AR exposure at ≥ 0.05 ppm was significantly associated with death due to severe notoedric mange, an ectoparasitic disease, and a precipitous population decline and genetic bottleneck occurred as a result of the mange outbreak from 2002-2006 (Riley et al. 2007, Serieys et al.

unpubl. manuscript). Notoedric mange was previously reported only in isolated cases in free-ranging felids (Pence et al. 1982, Maehr et al. 1995, Pence et al. 1995), although the disease may now be an increasing problem for bobcats across California (Serieys et al. 2013, Stephenson et al. 2013). All bobcats with severe mange tested for ARs were exposed (N = 19, Riley et al. 2007; N = 11, Serieys et al. 2013). These findings have led researchers to hypothesize that chronic, sublethal exposure to ARs may influence immune function in bobcats, increasing their susceptibility to severe mange infestation and their ability to mount an anti-mite response (Riley et al. 2007).

Following these findings of widespread exposure in multiple carnivore species in SMMNRA and the potential interaction between ARs and notoedric mange in bobcats, I initiated an investigation of risk factors for bobcat exposure to ARs in southern California. I took a novel approach to the study of AR exposure in wild animal populations by sampling both blood and liver samples to comprehensively detect exposure across a landscape of fragmented urban and large protected natural areas. Liver samples were collected postmortem to evaluate exposure history of individuals. Further, unlike previous studies, rather than relying exclusively on the opportunistic recovery of liver samples from carcasses to evaluate exposure, I also used blood samples collected antemortem during animal capture to evaluate recent exposure events. Therefore, I was able to significantly increase the number of samples collected as well as more intensively and strategically sample across my study area. I used multiple measures of AR exposure that included exposure prevalence both overall and to individual compounds, the number of compounds detected, and compound residue concentrations (toxicant load). Using these variable measures, I evaluated AR exposure over 16 years from 1997-2012 based on samples collected from 5 counties in southern California. I assessed risk factors for exposure

including sex, age, and season. I also assessed fine-scale spatial risk factors, including proximity to residential, commercial, and altered open areas. My primary goal in evaluating spatial risk factors for exposure was to pinpoint specific types of human development that may be the primary contributors to environmental contamination with ARs. Using a much larger number of samples collected over an extended period of time, than had been done previously by Riley et al. (2007), I examined the potential association between a range of residue concentrations and the number of compounds detected and notoedric mange. Finally, I compared SGAR exposure prevalence both before and after new United States Environmental Protection Agency (EPA) regulations took effect in June 2011, which were designed to decrease ecological risk associated with human use of these compounds (US EPA 2008).

I predicted differential rates of AR detection for different sample types. Specifically, I expected that higher exposure prevalence, and concentrations of compounds would be detected in liver samples than blood samples given that these compounds have longer persistence in hepatic tissue than blood (Vandenbroucke et al. 2008). However, I predicted that I would most frequently detect SGARs, instead of FGARs, in both blood and liver samples because SGARs are most frequently detected in hepatic tissue in California (Hosea 2000, Riley et al. 2007, McMillin et al. 2008, Gehrt and Riley 2010, Gabriel et al. 2012, Serieys et al. 2013). Over the course of the 16-year study period, I expected AR detection rates to increase concurrent with increasing urban development. I anticipated that males, more likely to utilize urban and altered open spaces than females, and with larger home ranges than females (Riley et al. 2010), would have greater AR exposure than females as a result of increased probability of contact with treated rodents. I predicted that older animals would have an accumulated risk of exposure, and thus have higher exposure prevalence and residue concentrations. Spatially, I expected that exposure

would be highly associated with animal proximity to human development, particularly residential and agricultural areas, and that animals residing in large protected natural areas would not be exposed to ARs. Finally, I anticipated that there would be an association between multiple AR exposure events measured as residue concentrations and the number of compounds detected in bobcats and severe notoedric mange.

METHODS

Study area and sample collection

Bobcat sampling primarily occurred during two field studies (Fig. 2.1). In Los Angeles and Ventura Counties, samples were collected by NPS and UCLA biologists from 1997-2012 during an ongoing NPS bobcat ecology study in SMMNRA (Riley et al. 2003, 2006, 2007, 2010, Serieys et al. 2013). The eastern boundary of SMMNRA is less than 10km from downtown Los Angeles and the park encompasses both large public regions of continuous protected habitat with minimal urban development, including state and national park lands, as well as highly fragmented areas with intense urban development. In the Orange County study area (OCSA), bobcats were sampled across a network of public nature reserves in a rapidly urbanizing landscape in, and surrounding, the Santa Ana Mountains, during a study by the U.S. Geological Survey (USGS) from 2006-2010 (Lyren et al. 2006, 2008). The Santa Ana Mountains straddle 3 counties that include Riverside, Orange, and San Diego but most of the samples (93%) were collected in Orange County. Human development across both study areas includes residential, commercial, agricultural development, and altered open areas such as golf courses and landscaped parks (Table 2.1). Samples were also opportunistically collected in two additional

areas north and south of my study areas in San Barbara and San Diego Counties when animals died in wildlife rehabilitation facilities or were reported dead by residents.

In SMMNRA, National Park Service biologists and I collected blood samples antemortem at the time of capture. Bobcats were captured with padded foothold traps (1996-1998) and cage traps (2000-2012) (Riley et al. 2003, 2006, 2007, Serieys et al. 2013). Animal capture, handling, and sample collection protocols were approved by the Office of Animal Research Oversight of the University of California, Los Angeles (Protocol ARC#2007-167-12) and by the Colorado State University Animal Care and Use Committee (Protocol #11-2453A). Protocols underwent extensive review in order to minimize animal stress and suffering. Scientific collecting permits were authorized through the California Department of Fish and Wildlife (SC-9791). From 2000-2009, the majority of trapping efforts occurred from mid-October to mid-February across sampling years, and thus the majority of blood samples were collected during the wet season. Animals were aged, sexed, weighed, measured, ear-tagged, and released at the capture site. A subset of individuals were also radiocollared as part of the ongoing NPS study (Riley et al. 2003, 2006, 2007, Serieys et al. 2013). To obtain serum samples, blood was centrifuged within 24 hours of collection and serum was collected. Blood and serum were stored at -20^{o}C or -80^{o}C until tested. Anticoagulant rodenticide compounds are stable (Waddell et al. 2013) and so the length of time in the freezer is not expected to affect the results.

In both study areas, I obtained liver samples during necropsies by veterinary pathologists (N = 23) or biologists (N = 146) from opportunistically found carcasses (e.g. road-kill) or from animals that died in rehabilitation centers. In SMMNRA, when radio-collared animals died, liver samples were also collected from these individuals whenever possible. For 20 individuals, blood was simultaneously obtained postmortem. The cause of mortality, collection date, sex, age class,

and location found were recorded. All animals were visually inspected for clinical signs of mange that included severe dermatitis, alopecia, and lichenification of the skin. If clinical mange was observed, skin scrapings in the affected areas were performed to identify mite species as previously described (Riley et al. 2007, Serieys et al. 2013, Stephenson et al. 2013). During necropsy, an upper canine tooth was extracted for quantitative age assessment, measured in years, using cementum annuli from a subset of individuals in both study areas (Matson's Laboratory LLC, Missoula, MT) (Crowe 1972). Capture and mortality locations were recorded using GPS devices. Liver was stored at -20^{o}C until tested.

Anticoagulant screen

I assessed residues, both presence and amount, of warfarin, coumachlor, bromadiolone, brodifacoum, diphacinone, chlorophacinone, and difethialone in 2 g of liver tissue, 1 g of serum, or 2 g of whole blood by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) as previously described (Riley et al. 2007, Waddell et al. 2013). Limits of quantitation for these anticoagulants vary according to their sensitivity to UV or fluorescence detection. In liver tissue these limits are 0.01 ppm for brodifacoum, 0.05 ppm for bromadiolone, warfarin, and coumachlor, and 0.25 ppm for chlorophacinone, diphacinone, and difethialone. In blood, limits of quantification were 1 ppb for each compound with method detection limits ranging from 0.28 to 0.45 ppb. ARs that were determined to be positive by LC-MS/MS, but were below the limit of quantitation by HPLC, were defined as above the limit of detection (LOD) or "above LOD".

Finally, to make comparisons between compounds detected in bobcat samples, and compound use in counties where bobcats were sampled, I accessed reported County (Los

Angeles, Orange, and Ventura Counties) pesticide use (measured in pounds) through the California Department of Pesticide Regulation online database (http://www.cdpr.ca.gov/docs/pur/purmain.htm) for the 4 most commonly detected compounds in bobcats. I accessed yearly records from 1997-2012. Records for Orange County were accessed only for the years for which I had samples from the study area (2006-2010). I averaged the pounds applied across the counties for each sample year (see Fig. 2.2c).

Land use analysis

To evaluate the surrounding landscape of all captured or recovered bobcats, I created buffer zones (circular polygons) with each capture or mortality location as the centroid. Each buffer zone area was equal to the size of a 95% minimum convex polygon home range consistent with the sex and sampling region of each individual (males: 5.2 km^2 SMMNRA; 5.6 km^2 OCSA; females: 2.3 $km^{2,}$ SMMNRA; 3.2 km^2 OCSA) (Riley et al. 2010). Animals that were sampled in Santa Barbara and San Diego Counties were excluded from land use analysis because exact sampling locations were unavailable. I clipped the 2005 land use dataset provided by Southern California Association of Governments (SCAG, http://gisdata.scag.ca.gov/Pages/Home.aspx) with bobcat buffer zones in ArcGIS 10.1 to quantify the land types used by each bobcat. Seventy-six land use variables were available for the bobcat buffer zones. These land use variables were broadly categorized into 5 general classes including: 1) agriculture; 2) commercial and industrial; 3) residential; 4) altered open areas such as landscaped parks, golf courses, and cemeteries; and 5) undeveloped natural areas (Table 2.1). To enhance my statistical power and to reduce the number of statistical tests and the probability of Type I errors, prior to statistical analyses, I merged the 76 SCAG land use variables into 13 groups based on similarity

and relevance to this study in terms of likely anticoagulant use (Tables B.1–B.3, Appendix). For each of the 4 general classes of anthropogenically developed land use type, I also used the total area of each class as a predictor variable. Thus, there were 17 spatial predictor variables used for analyses (Table 2.1). I quantified the percent cover of each of these 17 predictor variables in each buffer zone and used these land use data for analyses described below. To estimate the percent of each land use type within the study areas, I created a single minimum convex polygon surrounding all buffer zones separately for SMMNRA and OCSA. I then calculated the percent of each of the 17 land use variables within each study area's polygon (Table 2.1). Because golf courses and cemeteries share similar characteristics (manicured green lawns), and because cemeteries comprised < 0.20% of the study areas, cemeteries were grouped with golf courses and will be referred to only as golf courses below.

Data analysis

Descriptive statistics are presented as mean +/- standard deviation, median, and range. Proportion and exact 95% confidence intervals of anticoagulant exposure for males, females, adults, juveniles, and wet (November 1 – April 30) and dry (May 1 – October 31) seasons were calculated separately for blood and liver samples. For prevalence calculations using blood, recaptured animals were counted once and only the data from the most recent capture event were used. For spatial analyses using buffer zone data, all resampling events including recaptures and post-mortem sampling which occurred a minimum of 4 months apart were included in analyses. My rational for this approach was that evaluating risk factors for exposure was a primary goal of this study and I expect ARs in blood to decay faster than the minimum 4 month interval between captures (Eason et al. 2002, Erickson and Urban 2004, Vandenbroucke et al. 2008). Thus,

because successive samples of individuals are effectively independent measures of exposure, I avoided bias caused by multiple recaptures. For a subset of animals (N = 64) I had both liver and blood results. In this group, I combined the AR residue data for both tissue types to calculate the proportion and exact 95% confidence intervals of anticoagulant exposure, the range, and the mean and median number of compounds detected per individual.

I measured AR exposure using 11 variables for liver results, and one variable for blood results (Table 2.2). Using liver results, I evaluated total exposure as presence or absence of any compound as well as individual exposure to each of the 4 most commonly observed compounds (brodifacoum, bromadiolone, diphacinone, and difethialone). I also measured AR exposure as the total residue concentration in parts per million (ppm) of all compounds detected (total residues), as well as individually for each of the 4 most commonly detected compounds. Finally, I evaluated anticoagulant exposure as the total number of compounds detected in each sample (0-7). Using blood results, I evaluated total exposure only. The majority of detected ARs in blood were diphacinone, and the total concentration of ARs was quantifiable for less than 10% of samples tested (24% of positive samples).

I evaluated risk factors for AR exposure using three types of generalized linear models (GLM). Using a logistic regression, I evaluated risk factors for total exposure measured using blood and liver, and separately, for exposure to brodifacoum, bromadiolone, diphacinone, and difethialone based on liver samples. I used a log-linear GLM to evaluate risk factors for total residue concentrations and for residue concentrations for each of the 4 most commonly detected compounds in liver tissue. Two animals of 169 from OCSA had outlier residue concentrations of greater than 2 standard deviations above the mean, one for difethialone and the other individual for bromadiolone. These individuals were excluded from concentration analyses for these

specific compounds and for total residue analyses because preliminary analyses indicated that they dominated model results. I used a Poisson regression to evaluate risk factors for exposure to multiple compounds (0-7) for liver exposure data.

For each model type, I first performed univariate analyses to identify potential predictors, or risk factors, of exposure (Table 2.2). I tested land use categories within each individual buffer zone, study area (SMMNRA, OCSA), sex (male, female), age class (adult animals ≥ 2; juveniles < 2 years), age (in years), and season (wet, dry). To evaluate the change in detection rates over time, animals were grouped into 2-3 year increments depending on the number of animals sampled yearly such that in all time increments, $N \geq 7$ ($\overline{N} = 23$; Fig. 2.2). Four percent (N = 4) of liver samples were collected during 1997-1999, and due to limited samples size, this time increment was excluded from temporal analyses. For analyses involving SGARs (brodifacoum, bromadiolone, difethialone), I examined whether exposure to these compounds changed after the EPA regulations took effect in June 2011. Sixteen percent of liver samples (N = 28) were collected from August 2011-July 2012 after the EPA regulations took effect.

Next, I performed multivariate GLMs to test the influence of predictor variables on AR exposure while controlling for all significant predictor variables. The multivariate GLMs were selected by backward stepwise selection using Akaike's Information Criterion (AIC) for model selection. I selected and report the strongest models with ΔAIC values ≤ 2 (Burnham and Anderson 2002). I report β, the standard error of β, and 95% confidence intervals for β. A positive β indicates a positive association between the predictor and the exposure outcome, while a negative β indicates a negative association.

I also used logistic regression analyses to examine anticoagulant exposure parameters as predictors for clinical notoedric mange. My predictor variables for these analyses included the 11 anticoagulant exposure measures and the 17 land use predictors. Analyses were performed as above with univariate models followed by multivariate analyses. Using Fisher's exact tests, I examined the association between notoedric mange and anticoagulant exposure parameters including the total number of compounds (≥ 2, ≥ 3, and ≥ 4) and the threshold value of total residues ≥ 0.05 ppm suggested by Riley et al. (2007). To examine the potential relationship between mange and AR residues in more detail, I visualized the data by plotting the number of animals, both with and without mange, exposed to a range of anticoagulant residue concentrations (Fig. 2.3). I observe an increase in the number of mange cases at a residue range of 0.25 – 0.49 ppm. Consequently, I also used a Fisher's exact test to evaluate the association between mange and total residues ≥ 0.25 ppm. Next, I used a Kolmogorov-Smirnov test to evaluate the difference in the distribution of residue concentrations in bobcats that died with mange compared with those that died without mange. Finally, I used a Wilcoxon-rank sum test to evaluate the difference in median residue concentrations between the two groups.

Because frequent methods of correction for multiple tests have been described as overly conservative with a higher probability of generating Type II errors in comparison with Type I errors, particularly for ecological studies with low statistical power (Moran 2003), I did not correct for multiple tests. Thus, all statistical tests were considered significant when $\alpha \leq 0.05$. All statistical analyses were performed in the program R (R Development Core Team 2011).

When data on the sex (liver, N = 18; blood, N = 2), age class (liver, N = 25; blood, N = 3), year collected (liver, N = 7), season collected (liver, N = 7), or mange status (N = 13) were unavailable, these samples were excluded from prevalence estimates and statistical analyses

requiring these data. I also excluded exposure results from statistical analyses for livers from two fetuses, one from each study area, collected when their mothers were each hit by a car, and a liver from a one day-old kitten because their exposure was likely not independent from that of their mother.

RESULTS

Sampling

A total of 378 samples were collected from 304 animals. One hundred seventy-two livers were collected postmortem and 206 blood samples were collected from 195 individuals. In addition to the 20 individuals for which blood and liver samples were collected simultaneously during necropsy, blood was collected at the time of capture and liver samples were later recovered during necropsies for 44 radiocollared animals. Thus, I had a total of 64 blood and liver samples collected from the same individuals. Eleven blood samples from recaptured individuals were excluded from prevalence estimates (Table 2.3). One hundred four livers were collected in SMMNRA (Los Angeles County N = 44; Ventura County N = 55; N/A = 5) and 56 in OCSA (Orange County N = 52; Riverside County N = 1; San Diego County N = 3). Eleven livers from Santa Barbara County and San Diego County (outside of OCSA) were collected from individuals that died in rehabilitation centers (N = 9) or were reported dead by local residents (N = 2). One hundred eighty-nine blood samples were from SMMNRA (Los Angeles County N = 88; Ventura County N = 101), 16 were from OCSA (all Orange County), and one was from San Diego County. Age, in years, was available for 66 animals from SMMNRA and OCSA, and ranged from 0 to 12 years.

Prevalence of exposure

I observed widespread exposure of bobcats to anticoagulant rodenticide compounds. Using liver samples (N =169), 88% of samples had 1–5 compounds (Table 2.3; mean = 2.32, median = 2.00). The range of total residues detected in liver was 0.00 – 5.81 ppm (mean = 0.59, SD = 0.80, median = 0.40). The compounds most frequently detected were 3 SGARs that included bromadiolone, brodifacoum, and difethialone, and 1 FGAR, diphacinone. Mean values for the 4 most commonly detected compounds were: brodifacoum, 0.14 ppm (SD = 0.20); bromadiolone, 0.38 ppm (SD = 0.55); difethialone, 0.04 ppm (SD = 0.31); diphacinone, 0.03 ppm (SD = 0.12). Brodifacoum and bromadiolone were detected most frequently in liver samples (Fig. 2.4) and were detected approximately twice as frequently as difethialone or diphacinone. FGARs warfarin and chlorophacinone were rarely detected and coumachlor, another FGAR, was not detected in liver samples. Seventy-seven percent of all bobcats and 87% of those exposed showed the presence of ≥ 2 compounds in the liver (N = 169).

In contrast, 39% of blood samples tested positive for ARs (Table 2.3) ranging from 1–4 compounds (mean = 0.53 compounds, median = 0.00). In blood, only one compound was detected in 76% of cases. The total residues detected in blood ranged from 0 – 0.16 ppm (mean = 0.002, SD = 0.01, median = 0.00). Diphacinone, the most commonly detected compound in blood, was detected more than 3 times as frequently as brodifacoum or bromadiolone (Fig. 2.4). For animals with both blood and liver samples (N = 64), 92% were exposed, most frequently to three compounds (median = 3.00, mean = 2.61, range 1-5). For total residue concentrations in combined liver and blood results, I detected a range of 0.00 – 5.81 ppm (mean = 0.57, SD = 0.83, median = 0.39).

Percent exposure was similar across sexes, age classes, and age measured in years using liver or blood samples (Table 2.3). Two fetal bobcats were exposed to anticoagulant compounds. One animal was exposed to 2 compounds (brodifacoum and diphacinone) while the other was exposed to 5 compounds (brodifacoum, bromadiolone, diphacinone, difethialone, and chlorophacinone). For both fetuses, all compounds detected were above LOD but not quantifiable. The mother of the fetus with 5 compounds was also tested for exposure and had quantifiable levels of brodifacoum (0.32 ppm), bromadiolone (0.58 ppm) and above LOD for difethialone, diphacinone, and chlorophacinone. A day-old kitten that was tested was not found exposed to ARs. Bromadiolone was detected above LOD in the liver tissue of the mother, sampled approximately 3 months after the death of the kitten. This adult female lived in the most protected, natural region of SMMNRA where anticoagulant exposure was rarely documented. She was also in a rehabilitation facility where she had a known diet and was unexposed to anticoagulants for approximately one month prior to birthing the litter, so the absence of exposure in the kitten was not surprising.

Exposure did not vary by season when tested using liver samples (Table 2.3). In contrast, when tested using blood, animals were significantly more likely to be exposed during the dry season (OR = 2.58) compared with the wet season (Tables 2.4 and 2.5). Overall I detected 72% more exposure during the dry season than during the wet season with 32% exposure detected during the wet season, and 55% exposure detected during the dry season. I did not detect a trend, across sampling years of exposure prevalence in blood samples. Using liver samples, I examined exposure prevalence over time and found exposure to exceed 67% over all years indicating high exposure prevalence throughout the study (Fig. 2.2a). Exposure rates varied for each of 6 compounds across sampling increments (Fig. 2.2a; brodifacoum, 50-92%; bromadiolone, 17-

96%; diphacinone: 0-72%; difethialone, 16-35%; warfarin, 0-4%; chlorophacinone, 0-9%). Coumachlor was not detected in any liver tissue. Total exposure prevalence was highest during 2003-2004 and 2011-2012. There was significantly less total exposure and bromadiolone exposure in 2001-2002 compared with other years in the study (Table 2.5, Fig. 2.2a). Diphacinone exposure was significantly greater in 2003-2004 and 2011-2012 compared with other year increments (Table 2.5, Fig. 2.2a). However, both total and bromadiolone residue concentrations detected were greatest between 2005-2010, although the differences in the residue concentrations across time increments were not significant (Fig. 2.2b). These years included OCSA samples, where significantly greater bromadiolone residues were detected (Table 2.6, Fig. 2.5). Although the residue concentrations I detected in 2011-2012 were lower for all compounds detected, the difference in exposure and residue concentrations was not significant, and the apparent decrease in residue concentrations is the result of having OCSA samples only for the years 2006-2010 where bromadiolone residues were significantly higher (see below and Fig. 2.1b, Appendix). Further, the decrease in total and bromadiolone residues mirrors the County reports I complied of the number of rodenticide pounds applied across counties (Fig. 2.2c and Fig. B.1c, Appendix). Sixteen percent (N = 28) of my liver samples were collected from August 2011 to July 2012, after the new EPA requirements took effect. There were no significant differences in exposure rates or residue concentrations after the new EPA regulations took effect in August 2011 (post-regulation: brodifacoum 87%, 0.10 ppm; bromadiolone 82%, 0.27 ppm; difethialone 36%, 0.02ppm; compared with 2001-2010 in Fig. 2.2a-b and Fig. B.1a-b, Appendix).

Spatial correlates of exposure

For liver samples, buffer zone land use estimates were available for 121 of 169 animals, while for blood samples, buffer zone data were available for 196 of 206 samples. Exposure

prevalence, measured using liver tissue, did not significantly differ between SMMNRA (89%, 95% CI: 81 – 94; N = 104) and OCSA (84%, 95% CI: 71 – 92; N = 55) (Fig. 2.5). However, significantly higher concentrations of total residues, brodifacoum, and bromadiolone were detected in liver tissue from OCSA compared with SMMNRA (Fig. 2.5; Table 2.6). The mean total residues, even with two outliers removed, was 0.84 ppm for OCSA, compared with 0.40 ppm in SMMNRA. Brodifacoum was detected at significantly greater concentrations in liver tissue collected in OCSA (0.21 ppm) compared with SMMNRA (0.12 ppm). The mean concentration of bromadiolone detected in OCSA (0.63 ppm) was approximately three times the mean concentration detected in SMMNRA (0.22 ppm).

Landscape variables were important predictors of exposure in both blood and liver samples (Tables 2.5 and 2.6). Percent natural area in each individual buffer zone was negatively associated with exposure in blood as measured by the total number of compounds and total residues, and exposure in liver by brodifacoum and bromadiolone concentrations (Table 2.6). Golf courses and total altered open area were positively associated with exposure in blood (Table 2.5) and with total residues and the concentrations of bromadiolone and brodifacoum in liver (Table 2.6).

All 3 agricultural categories (Table 2.1) and total agricultural area were positively associated with exposure in blood (Table 2.5). The 3 agricultural categories included crops, pastures, orchards, and vineyards, horse ranches, and other agriculture that comprised specimen gardens, arboreta, abandoned orchards/vineyards, and nurseries (Table B.1, Appendix). However, brodifacoum exposure was negatively associated with crops, pastures, orchards, and vineyards (Table 2.5), and given that SGARs are restricted for use indoors and within 100 m from human structures, this negative association is not surprising . Commercial and industrial areas were

positively associated with bromadiolone and diphacinone concentrations detected in liver samples (Table 2.6). Water storage and transfer facilities and total commercial and industrial areas were positively associated with exposure in blood samples (Table 2.5). Office and retail area was positively associated with brodifacoum concentration in liver samples (Table 2.6).

Residential areas were frequently positively associated with AR values observed in both blood and liver samples (Tables 2.5 and 2.6). Multifamily high-density residential area was positively associated with exposure in blood and bromadiolone concentration in liver. Single-family high-density residential area was among the most frequent land use types to have positive associations with anticoagulant exposure measures. For 8 of 11 anticoagulant exposure models tested, the percent of single-family high-density residential area within the buffer zones was a significant predictor. Single-family high-density residential area was positively associated with exposure in blood and liver and the total number of compounds and total residues in liver samples. Single-family high-density residential was also positively associated with brodifacoum and diphacinone exposure and brodifacoum and bromadiolone concentrations in liver. Total number of compounds, total residues and concentrations of brodifacoum and bromadiolone measured in liver were positively associated with total residential area. Total residential area was associated with exposure detected in blood and liver and exposure to brodifacoum and diphacinone in liver.

Multivariate models

I found multivariate models best-suited to explain anticoagulant exposure measures that included total exposure detected in blood, diphacinone exposure, total residues and brodifacoum and bromadiolone residue concentrations (Table 2.7). The best-fit model for exposure detected in

blood included season and percent natural areas within buffer zones. Total residential area and the year sampled, specifically 2011-2012, were the most important predictors of diphacinone exposure detected in liver tissue. For total residues detected in liver tissue, the best-fit model included golf courses, single-family high-density residential, and OCSA as the most important risk factors. For brodifacoum concentration detected in liver tissue, office and retail, single-family high-density residential, and total altered open space were the three most important predictors of residue load. Finally, mixed commercial and industrial, golf courses, single-family high-density residential, and OCSA were the most important predictors of total bromadiolone concentration in liver tissue.

Anticoagulants and mange

Of those individuals found dead in the study areas (N = 169), 83 did not have mange and 70 died with severe clinical mange. For 16 of 169 individuals, their mange status was not recorded and so they were excluded from analysis. The median total residues for bobcats with mange was 0.52 ppm (mean = 0.65, SE = 0.06), while for bobcats that died without mange, the median total residues was 0.24 ppm (mean = 0.53, SE = 0.09). The median concentration of residues was significantly different between the two groups ($W = 2141.00$, $P = 0.005$) and the distribution of residue concentrations within the two groups also differed significantly ($D = 0.28$, $P = 0.004$). The median number of compounds observed was 3 (mean = 3.00) in bobcats with mange and 2 (mean = 2.00) for bobcats without mange. Sixty-four percent of bobcats without mange tested positive for ≥ 2 compounds, while 93% of bobcats with mange tested positive for ≥ 2 compounds.

Clinical mange was associated with anticoagulant exposure, brodifacoum exposure, brodifacoum concentration, difethialone exposure, and the total number of compounds detected. Additionally, clinical mange was positively associated with total residential area, but this was the only significant land use predictor (Table 2.6). The mean total residential area in mange bobcat buffer zones was 32.20% (SD = 18.61, median = 29.39) compared with a mean of 23.32% for bobcats without mange (SD = 19.60, median = 19.05). After controlling for multiple AR parameters and land use, brodifacoum and difethialone exposure remained significant predictors of clinical mange and land use was not a significant predictor of clinical cases of the disease (Table 2.7). I found a strongly significant association between mange and total residues ≥ 0.05 ppm and total residues ≥ 0.25 ppm (Fig. 2.3, Table 2.4). Bobcats that were exposed to ≥ 0.05 ppm were 4.0 times (95% CI: 1.67-10.48) more likely to die with severe notoedric mange than without, while those exposed to ≥ 0.25 ppm were 3.2 times (95% CI: 1.51-6.84) more likely to die with severe mange than without. Additionally, I observed a strong association between exposure to ≥ 2 compounds and clinical mange (Table 2.4). Specifically, bobcats were 7.3 times (95% CI: 2.55-25.70) more likely to die with severe mange than without if they were exposed to 2 or more AR compounds. There were also significant associations between mange and exposure to ≥ 3 and ≥ 4 compounds (Table 2.4).

Anticoagulants and mortality

Anticoagulant exposure detected in blood was significantly more frequent in samples collected postmortem compared with samples collected antemortem (Tables 2.4 and 2.6). In 75% of blood samples collected postmortem (N = 20), I detected at least one AR compound. When blood samples collected at the time of mortality were excluded from blood AR prevalence

estimates, I detected a 34% exposure prevalence in blood samples collected at the time of animal capture (N = 175) compared with 39% overall (N = 195). For blood samples collected at the time of mortality, ARs were detected in 77% of bobcats that died of mange (N = 13), 100% of bobcats that died of vehicle collision (N = 5), and a bobcat that died of starvation after a wildfire. Three bobcats that died of mange, one from a control action, and another that died of unknown cause did not have detectable ARs in their blood.

DISCUSSION

I documented widespread exposure of bobcats to first- and second-generation anticoagulant rodenticides. Bobcats are obligate carnivores that consume a wide range of small mammals (Anderson and Lovallo 2003) including mice, rats, and gophers (Fedriani et al. 2000, Riley et al. 2010) that are frequent targets of pest control campaigns within SMMNRA (Morzillo and Mertig 2011a, 2011b, Morzillo and Schwartz 2011, Bartos et al. 2012) and elsewhere (Morzillo and Mertig 2011b). Given that bobcats are obligate carnivores that generally eat live, or recently dead, prey and are not omnivores that eat fruits and nuts, it is very unlikely that they consume rodent baits directly. Thus bobcat exposure to ARs is likely to be predominantly, if not entirely, secondary. Exposure rates and compounds detected varied considerably by sample type, but in my most completely sampled animals having blood and liver data, I detected an exposure rate of 92% across the study areas, with animals most frequently exposed to 3 or more compounds. These findings are among some of the highest reported prevalence rates for AR exposure in a nontarget predatory species (e.g. Riley et al. 2003, Shore 2003, Fournier-Chambrillon et al. 2004, Walker et al. 2008, Gehrt and Riley 2010, Elmeros et al. 2011, Gabriel et al. 2012, Sánchez-Barbudo et al. 2012). Additionally, the combined liver and blood results

indicate that both exposure prevalence and exposure to multiple compounds, specifically diphacinone, may be underestimated with liver samples alone. I detected exposure to multiple compounds in two fetal bobcats, the first such cases, to my knowledge, reported for any wildlife species. These data indicate multiple exposure events occurring for bobcats across multiple regions in southern California, and suggest the potential for chronic exposure to ARs that can begin during prenatal development.

The toxicokinetics (the movement of toxic substances within the body) of ARs varies by compound, dose, and host species, and there are no specific toxicokinetic studies in wildlife species. For example, the amount of time required for the compound concentration in plasma to be reduced by one-half of the initial plasma concentration (plasma half-life) is reported to be 2-10 days for brodifacoum in dogs (Woody et al. 1992, Robben et al. 1998) compared with approximately 92 days in mice (Vandenbroucke et al. 2008). For domestic cats, to my knowledge, only LD50 levels of some compounds have been reported (Erickson and Urban 2004). However, hepatic half-lives for ARs are generally reported across multiple species to be significantly longer than plasma half-lives, particularly for SGARs (Kamil 1987, Robben et al. 1998, Petterino and Paolo 2001, Vandenbroucke et al. 2008). Of 8 anticoagulant compounds orally administered to laboratory mice, the hepatic half-lives of 5 compounds were 3-4 times longer than observed in plasma (Vandenbroucke et al. 2008). The toxicokinetics of secondary AR exposure is perhaps more complex because the movement of the residues in both the primary and secondary consumer must be considered (Erickson and Urban 2004). I am thus limited in my ability to interpret bobcat AR exposure results with respect to dose and time since exposure for either blood or liver sample data. However, because I most frequently detect diphacinone in

blood despite its having a shorter plasma half-life than SGARs (Erickson and Urban 2004), diphacinone may be the compound that bobcats encounter most frequently in SMMNRA.

Risk factors for exposure

Exposure prevalence detected using liver tissue was high throughout the course of the 16-year study, ranging from 67 to 100% for each 2- to 3-year time period, indicating high risk of AR exposure in bobcats since at least 1997. Despite increased awareness and concern for the impacts these compounds pose to nontarget wildlife in recent years (Erickson and Urban 2004, US EPA 2008), particularly in California (Gabriel et al. 2012, California Department of Pesticide Regulation 2013), my samples indicated an increase in overall exposure both in prevalence and residue concentrations since 2002. Specifically, I detected significant increases in total AR exposure, bromadiolone exposure, and total number of detected compounds. However, with the exception of diphacinone, overall exposure prevalence and exposure to individual compounds appears to have been relatively constant from 2003-2012. Total compound and bromadiolone residues detected were highest from 2005-2010, the time increments for which OCSA samples were available which reflects the degree of bromadiolone use in Orange County. Diphacinone exposure also increased in frequency over the course of my study, reaching a high in 2011-2012. Despite this increase, the quantity that was applied in each county as reported to DPR does not appear to have significantly changed over the course of the study (Figure 2.2c and Figure B.1c, Appendix). Thus, increased diphacinone exposure may be the result of increased use of the compound in residential areas by home owners and pest control companies that do not report the amount of ARs applied annually. In fact, single-family high-density and total residential area were important predictors of diphacinone exposure. Diphacinone is a first-generation compound

and is considered to pose less risk to nontarget wildlife than the more toxic SGARs (Erickson and Urban 2004), although the degree to which there are additive or interactive effects between diphacinone and second generation ARs is unknown. Further, FGARs still pose toxic effects to wildlife and secondary exposure can be a direct source of mortality for some species (Littrell 1988, Stone et al. 1999, Riley et al. 2003). Despite new restrictions in the U.S. that limit the sale and distribution SGARs that took effect in June 2011(US EPA 2008), I did not detect a decrease in SGAR exposure or residue concentrations since June 2011. However, given the long hepatic half-lives of SGARs, an insufficient period of time may have passed to detect the reduced environmental impact intended with the new regulations. Further, I have no evidence that SGAR availability decreased immediately after the June 2011. In many cases, there is an allowance to exhaust existing stock and a major SGAR manufacturer has appealed the EPA mitigation regulations.

Bobcats had a significantly higher risk of AR exposure during the dry season; I detected more than twice as much AR exposure during the dry season compared with the wet season. The risk of secondary poisoning for predatory species would be highest during peak efforts of rodent control and seasonal variation in AR concentration in wildlife have been found to coincide with variations in rodent population size and intensity of control efforts (Fournier-Chambrillon et al. 2004). In southern California, the dry season coincides with peak rodent activity (Meserve 1976), and residents in the region are known to use ARs to target rat, mice, squirrel, and gopher populations (Morzillo and Schwartz 2011, Bartos et al. 2012). Although I detected no seasonal differences in exposure in liver samples, the long hepatic half-lives of SGARS likely obscured my ability to detect seasonal differences. Additionally, because SGARs may persist in small mammal species from 90 to 135 days after removal of poison baits, poisoned small mammals

remain a continuing source of exposure for predatory species long after the end of poisoning programs (Murphy et al. 1998, Sage et al. 2008).

Because an accumulated risk of exposure may occur with animal age, and female bobcats have smaller home ranges and are less likely to use urban areas compared with males (Riley et al. 2003, 2010), I expected to detect demographic differences in AR exposure prevalence and residue concentrations. However, neither age nor sex significantly influenced the probability of exposure in my study areas. Sex differences in AR exposure have been observed in stoats (*Mustela erminea*) and because female stoats consume more small mammals than males, the dietary difference was hypothesized to explain higher AR exposure prevalence in females (Murphy et al. 1998, McDonald et al. 1998). Within my study areas, the prevalence of exposure was high and may obscure my ability to detect demographic differences in AR exposure. Further, the relatively high mobility of some rodent species may lead to AR exposure in even those individuals that avoid the use of urban areas. Both wood mice (*Apodemus sylvaticus*) and house mice (*Mus domesticus*) were found exposed to multiple AR compounds in Northern Ireland even though they were sampled in agricultural areas where ARs were not in use (Tosh et al. 2012). Thus, movement of individuals between areas where AR control efforts differ may occur (Tosh et al. 2012) and the risk of secondary AR exposure in predatory species may not be limited to areas where ARs are in use.

Spatial predictors of exposure

The significant association between AR exposure and individual land use types, particularly the percent of residential, commercial, and altered open areas within bobcat buffer zones, likely reflects the degree of AR use in those areas. Previous studies have found an

association between developed areas and AR exposure in nontarget wildlife. Ninety-five percent (N = 74) of wildlife carcasses sampled across California from 1994-1999 with exposure to ARs were reported to have been collected in areas with significant urban development (Hosea 2000). However, specific information about what type and the intensity of the urban development where individuals were sampled was not described in this study, although some animals were reported to have been sampled in recently urbanized areas adjacent to natural habitat. Previous studies found a significant positive association between total AR concentrations and the percent of bobcat (Riley et al. 2007) and mountain lion (Beier et al. 2010) radio-locations in areas altered by human development, including areas classified as altered open areas and areas of more intense urban development (e.g. composite residential, commercial, and industrial areas). These past studies were less specific in their examination of the influence of urbanization and land use on animal AR exposure. For example, Riley et al. (2007) categorized the landscape into three general categories (e.g., natural, altered open areas, or developed). Unlike these previous studies, I measured the influence of fine-scale spatial land use categories on anticoagulant exposure and found evidence of exposure across a wide variety of land type designations.

Single-family high-density residential (5 – 10 housing units/ha) and golf courses were among the most frequent risk factors for various measures of anticoagulant exposure in spite of comprising a relatively small percentage of the study areas (15.92% and 1.39%). Single-family high-density residential area was a significant predictor of exposure in 8 of 11 models as well as an important predictor of exposure in 3 of 5 best-fit multivariate spatial models (total, brodifacoum, and bromadiolone concentration). Total residential area was also an important predictor of diphacinone exposure. Although altered open areas were significant predictors of exposure in only 4 of 11 univariate AR exposure models tested, golf courses and total altered

area were significant risk factors in 3 of 5 best-fit multivariate models (total residues, and brodifacoum and bromadiolone residues), suggesting their importance as a risk factor for AR exposure and toxicant loads.

In a recent study in 2 southern California areas (SMMNRA, Bakersfield), residents in single-family high-density structures were the most likely to use ARs to control pest populations compared with multifamily or single-family low-density structures (Morzillo and Schwartz 2011). Further, resident AR use was highest in areas in close proximity to open areas, whether natural or altered open, compared with residential areas farther away from open spaces. Golf courses and other altered open spaces in the study areas are typically surrounded by, or very near to, single-family housing units. Of 21 golf courses in my study areas, 19 are bordered on at least 1 side by single-family high-density residential areas. Because AR use may be elevated in areas with altered open space in close proximity (Morzillo and Schwartz 2011), the high association between AR use and altered open areas may also be the result of increased AR use in the single-family residential areas nearby golf courses. In OSCA, where bobcats had significantly greater brodifacoum and bromadiolone residue loads, the mean percent of golf courses in bobcat buffer zones was nearly 5 times greater than in SMMNRA, potentially contributing to increased residue loads in OCSA. Overall, although these types of urban development may comprise a relatively small proportion (< 25%) of the study areas, Morzillo et al. (2011) has suggested that even a small degree of AR use in residential areas can lead to significant risk of exposure for wildlife. Further, both bobcats (Riley et al. 2010) and coyotes (Gehrt and Riley 2010) have been observed to routinely utilize residential and altered open areas such as golf courses, increasing their probability of exposure to ARs if the compounds are regularly used in these or nearby, areas.

The percent of natural habitat was negatively associated with both first- and second-

generation AR exposure and total residue concentrations detected. However, of 3 animals whose buffer zones were comprised of 100% natural habitat, I detected two with above LOD levels of bromadiolone in their liver tissue, and 1 individual with diphacinone residues in its blood. These data indicate that ARs may also affect wildlife that is found within protected park areas. Both of the individuals with bromadiolone residues were radio-collared during ongoing NPS research in SMMNRA, and their documented home ranges also did not extend beyond protected park boundaries (Riley et al. NPS unpubl.data). Previous NPS research on coyote utilization of urban areas found that even animals with the lowest urban association died directly from AR toxicosis (Riley et al. 2003). A recent study on fishers (*Martes pennanti*), a remote forest carnivore in protected undeveloped parkland in northern California, found 79% of fishers exposed to ARs and 4 died directly of toxicity (Gabriel et al. 2012). The authors hypothesized that illegal marijuana cultivation in remote areas was the source of ARs in their study area. Within SMMNRA, illegal marijuana cultivation is also a regular occurrence and so this may also be a source of AR exposure for animals that reside entirely in protected park areas.

Consequences of exposure

Although the prevalence of AR exposure was very high at 92%, AR exposure alone does not appear to be a significant source of direct mortality for bobcats, as there are very few cases of AR toxicosis in bobcats in the literature. None of the bobcats in OCSA died directly of anticoagulant toxicity, and in a broader study of poisoning cases of wildlife in California, Hosea (2002) observed clinical signs consistent with anticoagulant toxicosis in two bobcats, 1 of which was an individual from SMMNRA (Riley et al. 2007). During a bobcat ecology study in Marin County, a radio-collared bobcat died of anticoagulant toxicity and chlorophacinone was detected

in the liver tissue (Riley 1999). AR exposure was suspected to have caused gastrointestinal bleeding in bobcats that died of severe mange and were exposed to ARs, though other clinical signs of anticoagulant toxicity were absent (Serieys et al. 2013). Domestic cats are reported to be more tolerant of AR exposure than dog or rodents (Petterino and Paolo 2001, Erickson and Urban 2004). Whether this tolerance is similar for wild felids is unknown, but if so, it may account for the relatively few cases of toxicosis observed. However, felid tolerance to low-grade AR exposure may increase their vulnerability to any sublethal toxicosis.

In SMMRNA, secondary anticoagulant rodenticide exposure was associated with a population decline (Riley et al. 2007) and a genetic bottleneck (Serieys et al. unpubl. manuscript) that occurred due to notoedric mange (*Notoedres cati*). All 19 bobcats with advanced mange tested positive for ARs, and there was a statistical association between notoedric mange and AR residues ≥ 0.05 ppm (Riley et al. 2007). Notoedric mange and vehicle collisions are the primary sources of mortality for bobcats in the study areas (Riley et al. 2010, Serieys et al. unpubl.data, Riley et al. NPS unpubl.data, Boydston et al. USGS unpubl.data). Notoedric mange is an increasing problem for bobcats in California with cases now documented in 8 counties in northern and southern California. Across all of these areas, animals that died of notoedric mange were also found to be exposed to ARs whenever tests were conducted (Serieys et al. 2013, D. Clifford, pers.comm.). Interestingly, 67% (N = 110) of severe bobcat mange cases observed in central and southern California from 2002-2012 occurred during the dry season (Serieys et al. unpubl.data), which is coincident with increased AR exposure detected in blood samples. In my study, 69 of 70 bobcats that died with severe mange were exposed to ARs. With my enhanced sample size, multiple study areas, and longer time period, I also detected a strong association between total AR residues ≥ 0.05 ppm and notoedric mange and a significant association

between total residues ≥ 0.25 ppm and notoedric mange (Fig. 2.3). Finally, I also detected a strong association between exposure to ≥ 2 compounds and notoedric mange. Detection of multiple compounds and high residue concentrations in a single individual suggests multiple exposure events. Thus, a single anticoagulant exposure event itself may not increase bobcat susceptibility to mange, but rather repeated exposure events may be an important predictor of potential sublethal effects such as increased susceptibility to mange. These latter findings are perhaps more compelling evidence of an association between the disease and total anticoagulant residues ≥ 0.05 ppm. The proposed threshold of 0.05 ppm is equivalent to detection of AR exposure because the lower detection limits of some compounds, including bromadiolone are 0.05 ppm, while the detection limits of chlorophacinone, diphacinone, and difethialone are 0.25 ppm.

Severe mange in free-ranging wildlife and domestic animals is often associated with decreased immune competence (Pence and Ueckermann 2002), and humans that are immunocompromised are also more likely to suffer severe, crusted forms of mange due to infestation with a related mite, *Sarcoptes scabiei* (Walton et al. 2004, Roberts et al. 2005). The mode by which anticoagulant rodenticide exposure could compromise bobcat immunity is unknown, although recent studies in humans and on laboratory rats have shown therapeutic doses of warfarin to have both immunostimulatory and suppressive effects when administered for ≤ 30 days (Kurohara et al. 2008, Belij et al. 2012, Popov et al. 2013). Laboratory experiments have also shown that interactive effects between sublethal exposure to anticoagulants and other stressors can induce mortality. For laboratory rat and rabbit populations, sublethal anticoagulant doses produced 40-70% mortality when combined with other stressors, such as frostbite (Jaques 1959). Similarly, when stressed by shearing and captivity, merino sheep required lower doses of

the AR pindone to die as a result of anticoagulant toxicosis (Robinson et al. 2005). Recently, a potential interaction between the toxic effects of chlorophacinone and a bacterial pathogen, tularemia (*Francisella tularensis),* in common voles (*Microtus arvalis*) was described (Vidal et al. 2009). Voles that were infected with *F.tularensis* died at lower doses of chlorophacinone than uninfected voles. Tularemia prevalence was also higher in areas treated with chlorophacinone, and the authors suggested that the AR field treatment may have also facilitated the spread of the disease in the affected vole population.

Sublethal AR exposure may also negatively affect individuals directly and potentially have population-level effects if prevalence is high. In Denmark, there was a negative association between anticoagulant exposure and body condition in weasels (*Mustela nivalis*) and stoats (Elmeros et al. 2011). A reduced escape response has been observed in rats dosed with ARs (Cox and Smith 1992), and if carnivores secondarily exposed to ARs have a similarly reduced response to threats, they may be more vulnerable to vehicle collisions or predation. Elmeros et al. (2011) found that for both stoats and weasels, those that were sampled after being trapped had significantly lower total AR residue concentrations than those sampled after vehicle collisions and predation events. Although I have a limited sample size (N = 5), 100% of animals that died of vehicle collisions for which I collected blood postmortem had detectable AR residues in their blood (compared with 34% of captured animals), suggesting that recent AR exposure events potentially increases bobcat vulnerability to vehicle collision. Within SMMNRA, of 71 cases of animals that were killed by vehicles from 1996-2011, 60% of cases occurred during the dry season (Riley et al. NPS unpubl.data, Serieys et al. unpubl.data), which is also the season of increased AR detection.

Bobcats with severe mange also exhibit altered behavior that increases their susceptibility to other primary sources of mortality. For example, although bobcats are primarily nocturnal, especially in urban populations (Riley et al. 2003), bobcats with severe mange infestation are frequently observed wandering in urban areas during daylight hours (Riley et al. NPS unpubl.data, Serieys et al. unpubl.data). This shifted activity pattern likely increases their risk of being hit by vehicles, and one severely mangy bobcat was killed after wandering into traffic on a busy freeway off-ramp. This vulnerability to other sources of mortality secondary to severe mange could be exacerbated if these bobcats were also recently exposed to ARs. Specifically, I observed that 77% of bobcats with severe mange (N = 13), compared with 34% of captured bobcats, had detectable AR residues in their blood. Of 3 cases of bobcat predation by domestic dogs in SMMNRA, each was a bobcat with severe notoedric mange (Riley et al. NPS unpubl.data).

Our findings of fetal AR transfer raise the question whether there may be potential reproductive consequences for bobcats. Contaminant exposure that interferes with the reproductive success of wildlife populations may be a critical conservation issue since this exposure can lead directly to population declines. I tested two bobcat fetuses, 1 from each study area and both were exposed to multiple AR compounds with 1 exposed to 5 compounds. Reproductive consequences associated with AR exposure in other species have included increased probability of miscarriage, fetal toxicosis, fetal congenital deformities, and decreased sperm counts in humans (Ginsberg and Hirsh 1989), dogs (*Canis familiaris,* Munday and Thompson 2003), and sheep (*Oves aries*, Robinson et al. 2005). Fetuses are considered more susceptible to maternal anticoagulant toxicosis (Munday and Thompson 2003), because placental transfer is the only source of Vitamin K for the developing fetus (Winckel et al. 2008). In

humans, prenatal exposure to coumarin at therapeutic doses has been associated with abortions, stillbirths, neonatal deaths, and increased risk of central nervous system abnormalities that included both physical and developmental defects (Ginsberg and Hirsh 1989, Wesseling et al. 2001). Brodifacoum toxicosis was documented in neonatal puppies even though the mother was exposed four weeks prior to birth (Munday and Thompson 2003). AR exposure may also continue during early postnatal development via milk transfer (Bullard et al. 1975), although the impacts on postnatal development are unknown. AR exposure may be an important challenge for population viability in urban areas if chemical contamination creates detrimental environments for reproduction.

Conservation and management implications

Exposure of nontarget wildlife to ARs is increasingly recognized as a widespread conservation issue (Erickson and Urban 2004, US EPA 2008, California Department of Pesticide Regulation 2013) and numerous species have been documented as being exposed, sometimes causing direct mortalities (Scheuhammer 1987, Peakall 1992, Eason et al. 2002, Erickson and Urban 2004, Riley et al. 2007, Gabriel et al. 2012). Species that are exposed include federally endangered species such as San Joaquin kit foxes (McMillin et al. 2008), bald eagles (*Haliaeetus leucocephalus*; Stone et al. 2003, Salmon 2010), and the Northern spotted owl (*Strix occidentalis caurina*; Erickson and Urban 2004). Indirect mortalities associated with the poisons may also pose an important threat for wildlife populations, particularly those that are threatened or in recovery. For example, during a recent study of California fishers, a species that is presently a candidate for protection under the US Endangered Species Act, a lactating female died directly of anticoagulant toxicosis which most likely led indirectly to the death of her litter of kits

(Gabriel et al. 2012). For threatened populations, exposure to ARs may influence their reproductive success, lead to sublethal and lethal consequences and increase their vulnerability to other sources of mortality.

Although some U.S. States, such as California, are taking steps to increase regulation of the use and the availability of these poisons to consumers, these steps may be inadequate. SGARs are currently limited by regulation to use indoors or within 30 m (100 feet) of buildings and in California, the Department of Pesticide Regulation has proposed to reduce that distance to a 17 m (50 ft) radius from buildings. However, Tosh et al. (2012) found no relationship between distance from buildings and residue concentrations in two species of mice because of the high mobility of the small mammals even after ingestion of the poisons. They also detected a contaminated wood mouse (*Apodemus sylvaticus*) 110 m from a building where usage occurred and another 160m from a building where no usage occurred (Tosh et al. 2012). In residential areas within SMMNRA, residents have reported off-label use of ARs, placing SGARs up to 100 m from buildings (Bartos et al. 2012) and I have observed containers of SGARs in natural areas behind homes at greater than 30 m from a building. Residents who use ARs have also reported continued use of the compounds although they were aware of the threat the compounds posed to nontarget wildlife (Morzillo and Mertig 2011a). Thus, in order to reduce ecological risks associated with these compounds, more restrictive regulations, particularly regarding use in residential areas where wildlife may be especially likely to be exposed may be necessary.

TABLES AND FIGURES

Table 2.1. Classification of predictor land use variables used for analysis of dependent AR exposure measures. The percent of each land use within a single polygon drawn around all bobcat buffer zones for each study area and the mean across both study areas is shown. Additionally, the mean value of each land use type across bobcat buffer zones for each study area and across all composite bobcat buffer zones is shown. The sum of land-use variables for each study area do not equal 100% because some land-use types (e.g. open water, roads, railroads), comprising a mean of 0.55% of the study areas, were not included in analyses.

		Percent of study areas			Percent of buffer zones		
Broad classification	**Specific land use tested in models**	**SMMNRA**	**OCSA**	**Mean**	**SMMNRA**	**OCSA**	**Mean**
Agriculture	Crops, pastures orchards and vineyards	3.39	3.00	3.20	2.18	1.62	2.07
	Horse ranches	0.53	0.23	0.38	0.53	0.58	0.54
	Other agriculture	0.50	0.89	0.70	1.33	1.56	1.38
	Total agriculture	4.42	4.12	4.27	4.04	3.76	3.99
Commercial and industrial	Schools and religious	1.04	1.61	1.33	0.57	2.15	0.88
	Office and retail	1.29	2.89	2.09	1.18	1.20	1.18
	Mixed commercial and industrial	1.61	5.20	3.41	1.85	3.64	2.20
	Water facilities	0.34	0.57	0.46	0.49	3.79	1.13
	Total commercial and industrial	4.28	10.27	7.28	4.09	10.78	5.39
Residential	Multifamily/commercial high-density (> 25 units/ha)	1.38	4.55	2.97	2.14	4.00	2.50
	Single-family high-density (5 – 10 units/ha)	14.80	17.04	15.92	10.76	7.58	10.14
	Single-family low-density (< 5 units/ha)	5.63	1.96	3.80	3.90	8.22	4.74
	Total residential	21.81	23.55	22.68	16.80	19.80	17.38
Altered open space	Golf courses and cemeteries	1.02	1.75	1.39	0.55	2.67	0.96
	Other recreational/altered open space	0.61	1.43	1.02	0.53	1.86	0.79
	Total altered open space	1.63	3.18	2.41	1.08	4.53	1.75
Natural	Undeveloped natural	66.82	58.82	62.82	54.08	23.32	48.01

Table 2.2. Classification of variables used for model testing. Spatial, demographic, temporal, and seasonal variables were tested as predictors of various measures of anticoagulant exposure. We also tested the various anticoagulant exposure measures as predictors of severe, clinical mange. Refer to Table 2.1 for specific land use variables used in model testing. Total exposure was used as a predictor variable for exposure detected in blood and liver. All other anticoagulant exposure measures were used as predictor variables for exposure detected in liver only.

Classification	**Specific variables tested in models**
Spatial	Land use variables
	Study areas (SMMNRA, OCSA)
Demographic	Sex (male, female)
	Age class (adult, juvenile)
	Quantifiable age (years)
Temporal and seasonal	Season (wet, dry)
	Sample year 1996-2012 (2-3-year increments)
	Implementation of EPA regulations (pre-, post- June 2011)
Disease	Mange (yes, no)
Anticoagulant exposure measures	Total exposure (yes, no)
	Brodifacoum exposure (yes, no)
	Bromadiolone exposure (yes, no)
	Diphacinone exposure (yes, no)
	Difethialone exposure (yes, no)
	Total concentration (sum of all AR residues; ppm)
	Brodifacoum concentration (ppm)
	Bromadiolone concentration (ppm)
	Diphacinone concentration (ppm)
	Difethialone concentration (ppm)
	Number of compounds detected (0-7)

Table 2.3. Proportion (Prop.) and 95% confidence intervals of anticoagulant exposure across the study areas. Prevalence is partitioned by sample type, sex, age class, and season. When information on sex, age class, or season collected was not available, those data were not included in the proportion estimates, and so data partitioned by sex, age class, and season may not sum to the total number of blood or liver samples.

		Liver			**Blood**		
Variable	Group	N	Prop.	95% CI	N	Prop.	95% CI
	All	169	0.88	0.82 - 0.92	195	0.39	0.32 - 0.46
Sex	Female	77	0.88	0.78 - 0.94	86	0.38	0.28 - 0.50
	Male	74	0.89	0.79 - 0.95	107	0.40	0.31 - 0.50
Age class	Adult	107	0.91	0.83 - 0.95	127	0.40	0.32 - 0.49
	Juvenile	37	0.86	0.70 - 0.95	65	0.37	0.26 - 0.50
Season	Wet	96	0.90	0.81 - 0.95	139	0.32	0.25 - 0.41
	Dry	66	0.89	0.81 - 0.94	56	0.55	0.42 - 0.68

Table 2.4. Results of Fisher's exact tests for parameters that were significant during univariate GLM analyses.

Sample Type	Parameter	Comparison	Odds Ratio	95% Confidence Interval	*P*
Liver	Total residues ≥ 0.05 ppm	Severe mange *vs*. no mange	4.00	1.67 – 10.48	<0.001
	Total residues ≥ 0.25 ppm	Severe mange *vs*. no mange	3.16	1.51 – 6.84	<0.001
	Exposure to ≥ 2 AR compounds	Severe mange *vs*. no mange	7.27	2.55 – 25.70	<0.001
	Exposure to ≥ 3 AR compounds	Severe mange *vs*. no mange	2.11	1.06 – 4.23	0.023
	Exposure to ≥ 4 AR compounds	Severe mange *vs*. no mange	3.98	1.54 – 11.26	0.002
Blood	Exposure detected	Dry season *vs*. wet season	2.58	1.31 – 5.14	0.004
	Exposure detected	Capture event *vs*. mortality	5.55	1.80 – 20.49	0.001
	Exposure detected	Capture event *vs*. vehicle mortality	∞	1.00 – ∞	0.006

Table 2.5. Significant predictors of presence or absence of exposure in blood and liver. Only results from statistically significant univariate analyses are shown.

Outcome	Predictors of exposure		β	β SE	β 95% CI	*P*
Total exposure (blood)	Dry season		0.95	0.32	0.31 – 1.56	0.003
	Crops, pastures, orchards and vineyards		4.85	2.08	0.98 – 9.21	0.015
	Horse ranches		88.75	36.10	21.90 – 166.11	0.011
	Other agriculture		15.46	7.29	1.63 – 30.67	0.029
	Water transfer and storage facilities		93.63	36.16	29.58 – 174.10	0.006
	Golf courses		15.69	7.75	0.50 – 30.88	0.043
	Multifamily high-density residential		9.47	3.56	2.49 – 16.44	0.008
	Single-family high-density residential		1.87	0.88	0.14 – 3.60	0.035
	Total residential		4.36	1.80	1.01 – 8.02	0.016
	Total commercial/industrial		4.42	1.84	0.81 – 8.02	0.016
	Total altered open		17.17	6.63	2.43 – 49.17	0.010
	Total residential		2.61	0.82	1.01 – 4.20	0.001
	Natural		-3.41	0.68	-4.74 – -2.09	<0.001
Total exposure (liver)	Single-family high-density residential		7.58	3.45	0.81 – 14.34	0.028
	Total residential		6.05	2.29	1.56 – 10.53	0.008
	Year (2011-2012 reference)	2001-2002	-2.72	1.18	-5.03 – 5.51	0.021
Brodifacoum exposure	Crops, pastures, orchards and vineyards		-5.62	2.67	-10.87 – -0.38	0.036
	Single-family high-density residential		6.19	2.36	1.56 – 10.82	0.009
	Total residential		6.68	1.90	2.95 – 10.41	<0.001
Bromadiolone exposure	Year (2011-2012 reference)	2001-2002	-1.54	0.67	-2.91 – -0.17	0.022
Diphacinone exposure	Single-family high-density residential		2.31	1.12	0.11 – 4.51	0.039
	Total residential		2.07	0.99	0.14 – 4.01	0.035
	Year (2011-2012 reference)	2001-2002	-1.46	0.7	-2.83 – -0.09	0.036
		2005-2006	-1.67	0.62	-2.98 – -0.52	0.005
		2007-2008	-1.30	0.48	-2.34 – -0.42	0.005
		2009-2010	-0.94	0.56	-2.26 – -0.01	0.048

Table 2.6. Significant predictors of AR residue concentrations, total compounds detected, notoedric mange, and exposure detected in blood at the time of capture *vs*. mortality.

Outcome	**Predictor variables**		β	β SE	β 95% CI	***P***
Total concentration	Golf courses		5.88	1.01	3.90 – 7.85	<0.001
	Single-family high-density residential		1.24	0.46	0.34 – 2.13	0.008
	Total altered open		5.66	0.98	3.74 – 7.58	<0.001
	Total residential		1.31	0.44	0.44 – 2.17	0.004
	Natural		-1.20	0.35	-1.88 – -0.52	0.001
	Study area: OCSA		0.74	0.17	0.41 – 1.08	<0.001
Brodifacoum concentration	Office/retail		5.13	1.17	2.84 – 7.42	<0.001
	Golf courses		4.16	1.45	1.30 – 7.20	0.006
	Single-family high-density residential		1.31	0.54	0.25 – 2.37	0.017
	Total altered open		4.28	1.42	1.49 – 7.07	0.003
	Total residential		1.31	0.53	0.28 – 2.34	0.014
	Natural		-0.93	0.42	-1.75 – -0.11	0.029
	Study area: OCSA		0.58	0.22	0.11 – 0.96	0.014
Bromadiolone concentration	Mixed commercial/industrial		5.10	1.29	2.57 – 7.63	<0.001
	Golf courses		7.45	0.95	5.59 – 9.30	<0.001
	Multifamily high-density residential		1.58	0.76	0.09 – 3.08	0.040
	Single-family high-density residential		1.38	0.52	0.36 – 2.39	0.009
	Total commercial/industrial		1.43	0.57	0.31 – 2.55	0.014
	Total altered open		7.16	0.92	5.36 – 8.96	<0.001
	Total residential		1.38	0.51	0.38 – 2.39	0.008
	Natural		-1.45	0.40	-2.24 – -0.67	<0.001
	Study area: OCSA		1.03	0.21	0.61 – 1.45	<0.001
Diphacinone concentration	Mixed commercial/industrial		8.90	3.20	2.62 – 15.17	0.006
Total compounds	Single-family high-density residential		0.80	0.32	0.16 – 1.43	0.014
	Total residential		0.92	0.29	0.35 – 1.49	0.002
	Natural		-0.47	0.22	-0.90 – -0.03	0.036
	Year (2011-2012 reference)	2001-2002	-0.57	0.24	-1.04 – -0.10	0.018
Mange	Exposure		1.90	0.78	0.37 – 3.43	0.015
	Brodifacoum exposure		1.74	0.52	0.71 – 2.76	0.001
	Brodifacoum concentration		1.84	0.89	0.08 – 3.59	0.040
	Difethialone exposure		1.16	0.39	0.39 – 1.92	0.003
	Total compounds		0.56	0.15	0.26 – 0.85	<0.001
	Total residential		2.38	1.01	0.39 – 4.37	0.019
Mortality	Exposure (blood)		1.72	0.54	0.67 – 2.78	0.001

Note: Only results from statistically significant univariate analyses are shown.

Table 2.7. Results of the best-supported statistically significant multivariate model analyses for anticoagulant exposure and mange.

Outcome	Best-supported model	Predictor variables	β	β SE	β 95% CI	*P*
Total exposure (blood)	Season + Natural	Dry season	0.71	0.35	0.02 – 1.40	0.043
		Natural	-3.29	0.68	-4.62 – -1.95	<0.001
Diphacinone exposure	Total residential + Year	Total residential	2.57	1.12	0.37 – 4.77	0.022
		2001-2002	-1.62	0.82	-3.23 – -0.02	0.048
		2003-2004	-1.42	0.69	-2.77 – -0.62	0.040
		2005-2006	-2.11	0.80	-3.68 – -0.55	0.008
		2007-2008	-1.78	0.65	-3.06 – -0.50	0.006
		2009-2010	-1.43	0.72	-2.84 – -0.01	0.048
Total concentration	Golf courses + Single-family high-density residential + Study area	Golf courses	3.91	1.06	1.84 – 5.98	<0.001
		Single-family high-density residential	0.99	0.43	0.15 – 1.82	0.022
		OCSA	0.69	0.20	0.30 – 1.07	0.001
Brodifacoum concentration	Office/retail + Single-family high-density residential + Total altered open	Office/retail	4.49	1.09	2.34 – 6.63	<0.001
		Single-family high-density residential	1.22	0.55	0.15 – 2.29	0.027
		Total altered open	3.88	1.55	0.85 – 6.91	0.013
Bromadiolone concentration	Mixed commercial/industrial + Golf courses +Single-family high-density residential + Study area	Mixed commercial/industrial	3.48	0.99	1.55 – 5.42	0.001
		Golf courses	5.69	0.93	3.87 – 7.51	<0.001
		Single-family high-density residential	1.24	0.42	0.42 – 2.06	0.004
		OCSA	0.90	0.25	0.42 – 1.38	<0.001
Mange	Difethialone exposure + Brodifacoum exposure	Brodifacoum exposure	1.54	0.53	0.49 – 2.58	0.004
		Difethialone exposure	0.93	0.40	0.14 – 1.72	0.021

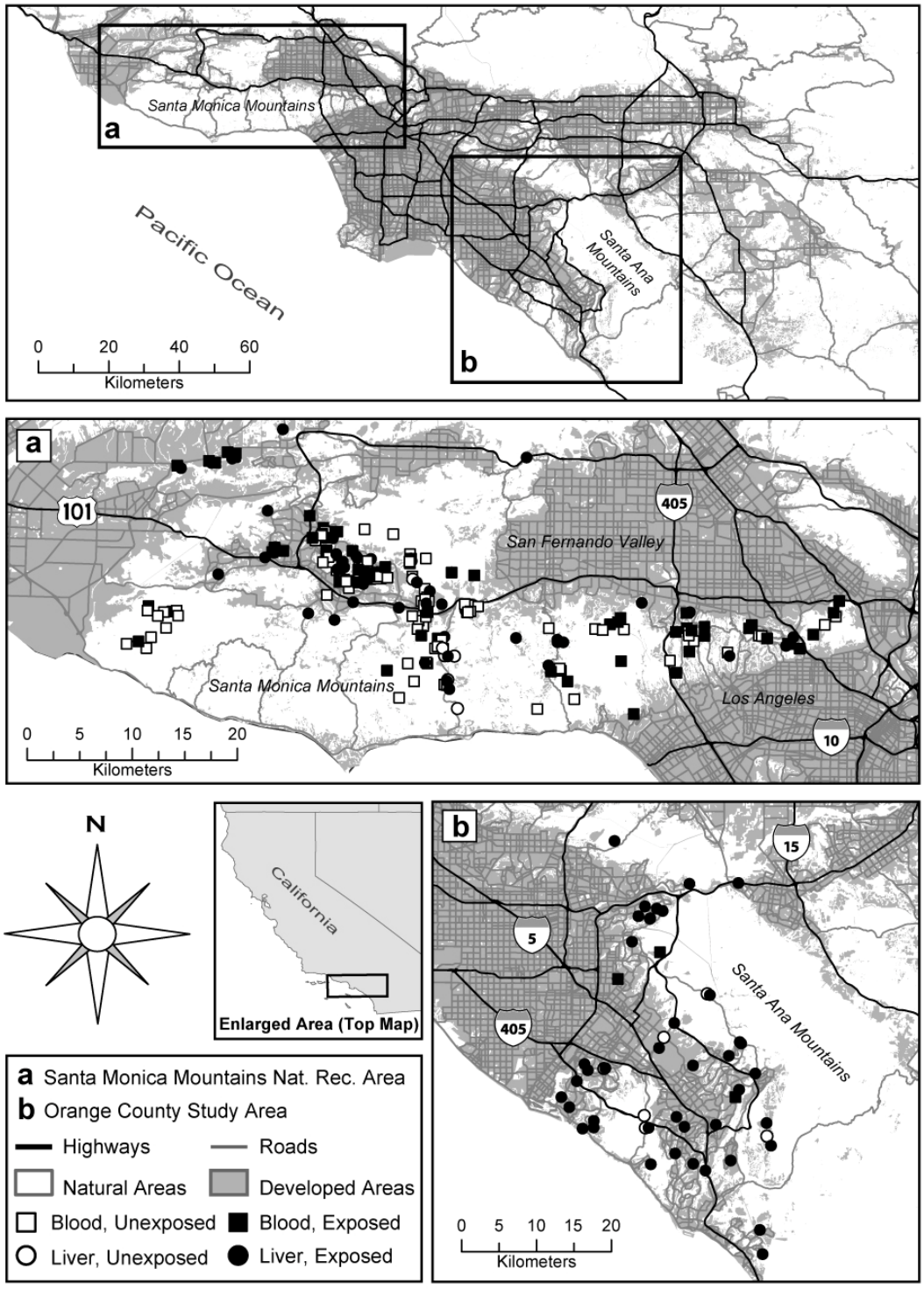

Figure 2.1. Map of the study areas. a) Santa Monica Mountains National Recreation Area (SMMNRA) and b) Orange County Study Area (OCSA). Sampling locations and exposure results are shown. Blood sampling locations are represented with squares while liver sampling locations are represented with circles.

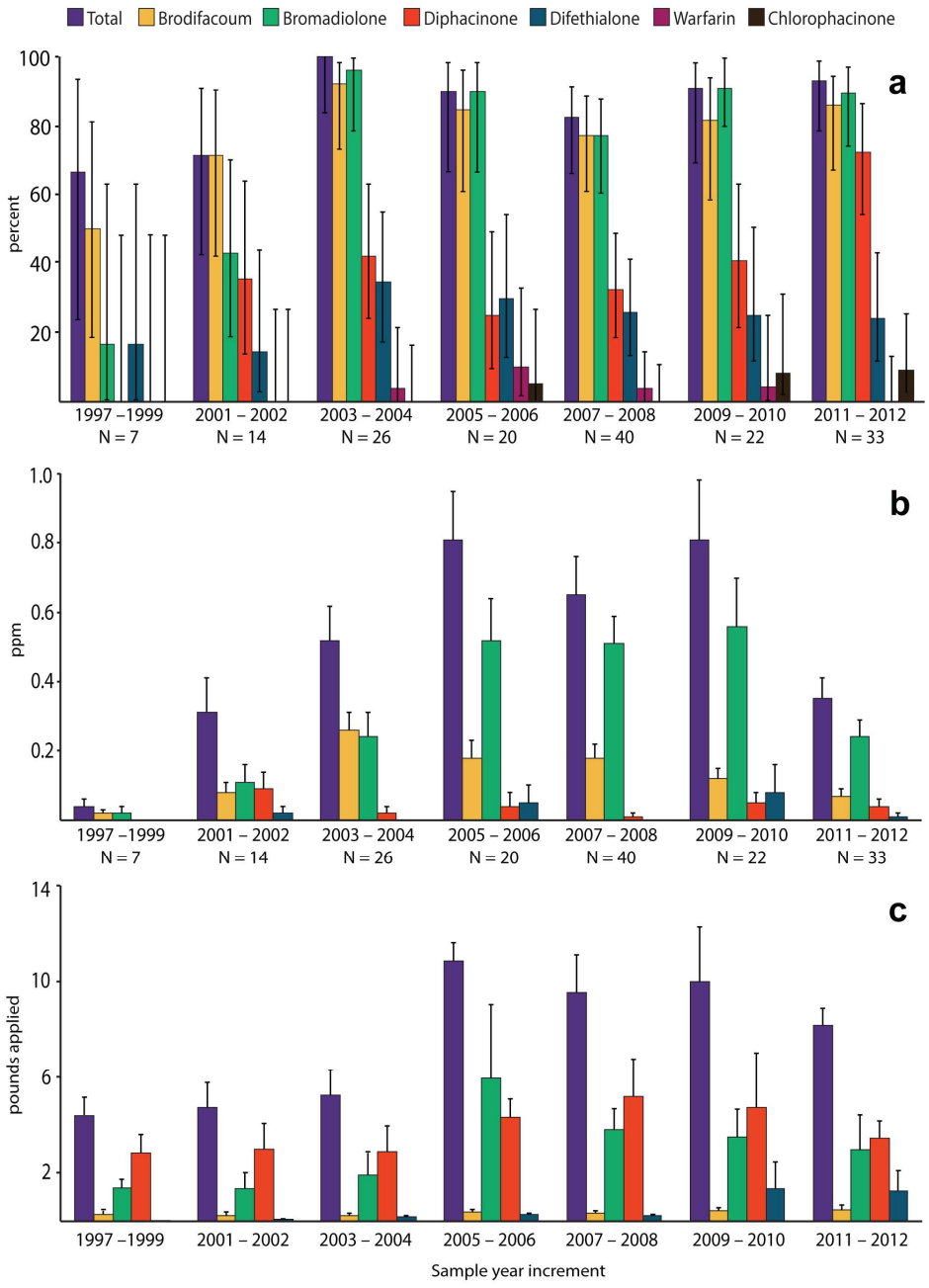

Figure 2.2. AR data across 2-3 year time increments. a) Exposure prevalence overall and by compound per 2-3 year increment. Error bars represent 95% confidence intervals. b) Concentrations detected per 2-3 year increments. Error bars represent standard errors. Warfarin, chlorophacinone, and coumachlor were rarely detected, and if so, were detected at above LOD levels (with the exception of chlorophacinone from 2006-2006 when 0.03 ppm was detected). Although lower concentrations of compounds were detected in 2011-2012, the difference, in comparisons with sample years from 2003-2010 was not significant. c) Reported pounds of each compound applied per year increment in the 3 primary study area counties. Error bars represent standard errors. Los Angeles and Ventura Counties are represented across all years and Orange County data was included from 2006-2010.

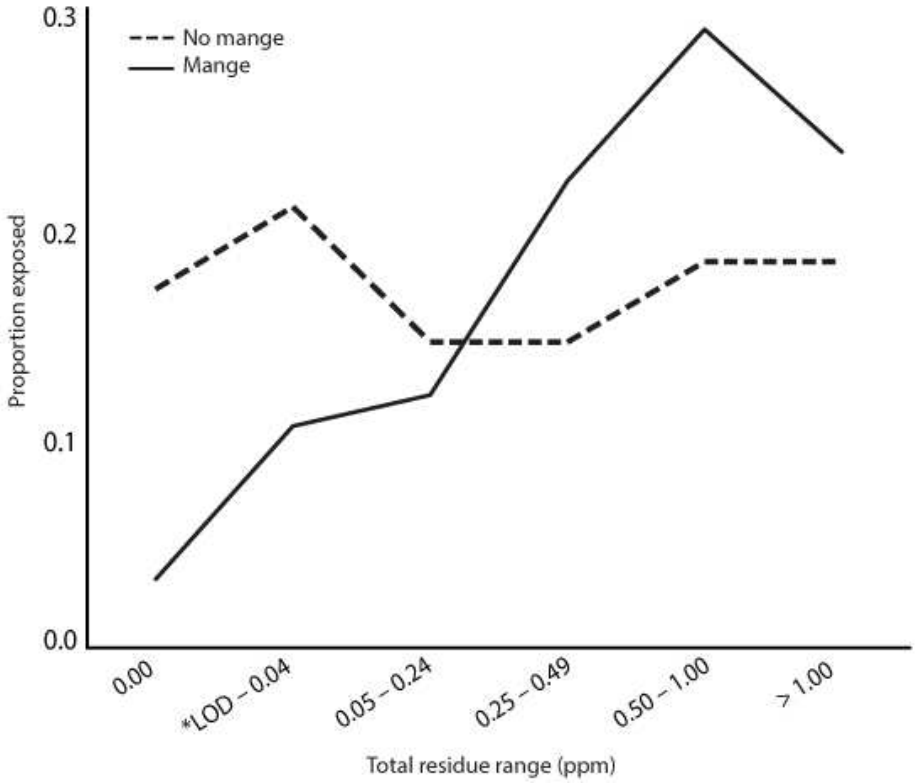

Figure 2.3. The proportion of bobcats that died with and without severe mange when exposed to a range of total anticoagulant residues (ppm). The proportion of mange cases, compared with bobcats without mange increases in the range of 0.25 – 0.49 ppm, and thus we investigated the relationship between mange and total residues ≥0.25 ppm. *LOD = the limit of detection. The limits of detection vary by compound. For brodifacoum and bromadiolone, the detection limits are 0.05 ppm, while the detection limits of chlorophacinone, diphacinone, and difethialone are 0.25 ppm.

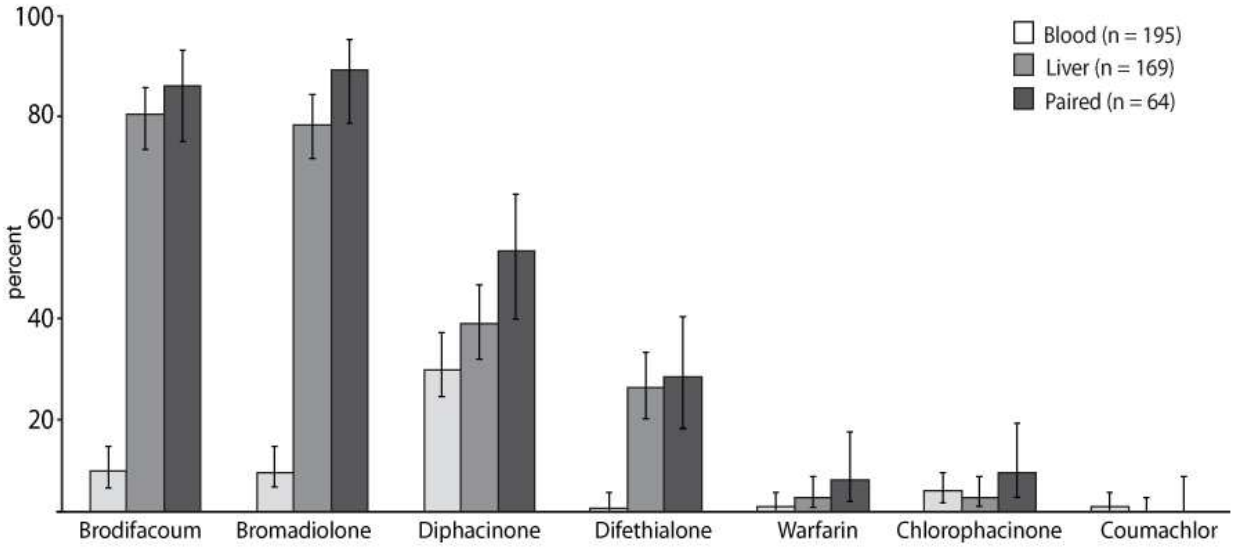

Figure 2.4. Detection prevalence of each anticoagulant compound in the liver, blood, and for a subset of individuals, paired blood and liver tissue results are provided.

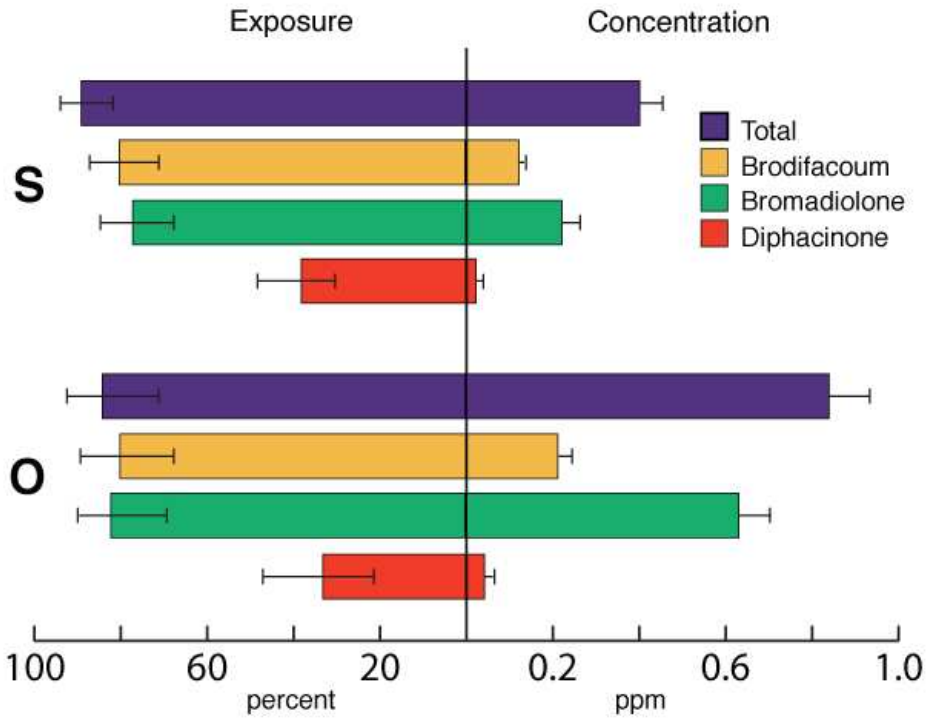

Figure 2.5. Left: Percent total exposure and exposure to individual compounds in SMMNRA (S) and OCSA (O). Bars represent 95% confidence intervals. Right: Total residue concentration and residue concentrations for each compound. Bars represent standard errors.

APPENDIX

Table B.1. Land use layers comprising the broad categories agriculture, altered open areas, and natural areas. The SCAG land use layers were merged based on similarity and relevance to this study.

Broad category	Merged name	Land use description (SCAG)
Agriculture	Crops, pastures orchards & vineyards	Irrigated cropland and improved pasture land
		Non-irrigated cropland and improved pasture land
		Orchards and vineyards
	Horse ranches	Horse ranches
	Other agriculture	Specimen gardens and arboreta
		Other agriculture
		Abandoned orchard/vineyard
		Nurseries
Altered open areas	Golf courses	Cemeteries
		Golf courses
	Other altered park	Developed local parks and recreation
		Developed regional parks and recreation
Natural (No expected AR use)	Undeveloped Natural	Other open space and recreation
		Undeveloped regional parks and recreation
		Vacant undifferentiated
		Vacant with limited improvements
		Wildlife preserves and sanctuaries
		Beach parks

Table B.2. Land use layers comprising the broad commercial and industrial category. The SCAG land use layers were merged based on similarity and relevance to this study.

Merged name	Land use description (SCAG)
Schools and religious	Elementary schools
	Junior or intermediate high schools
	Pre-schools/day-care centers
	Senior high schools
	Trade schools and professional training facilities
	Colleges and universities
	Religious facilities
Office and retail	Government offices
	Low- and medium-rise major office use
	Modern strip development
	Motion picture and television studio lots
	Older strip development
	Police and sheriff stations
	Regional shopping center
	Retail centers (non-strip with contiguous interconnected off-street parking)
	Fire stations
	Government offices
	High-rise major office use
Mixed commercial and industrial	Commercial recreation
	Commercial storage
	Communication facilities
	Electrical power facilities
	Liquid waste disposal facilities
	Maintenance yards
	Manufacturing, assembly, and industrial services
	Mineral extraction - other than oil and gas
	Mixed commercial and industrial
	Open storage
	Other public facilities
	Other special use facilities
	Research and development
	Under construction
	Wholesaling and warehousing
	Solid waste disposal facilities
Water facilities	Improved flood waterways and structures
	Water storage facilities
	Water transfer facilities

Table B.3. Land use layers comprising the broad residential category. The SCAG land use layers were merged based on similarity and relevance to this study.

Merged name	Land use description (SCAG)
Multifamily/commercial high-density (>25 units/ha)	Duplexes, triplexes and 2- or 3-unit condominiums and townhouses
	High-rise apartments and condominiums (>40 units/ha)
	Low-rise apartments, condominiums, and townhouses (25-40 units/ha)
	Medium-rise apartments and condominiums (>40 units/ha)
	Mixed multifamily-residential (duplexes, triplexes, apartments, condominiums, and/or townhouses)
	Hotels and motels
	Special care facilities
	Correctional facilities
Single-family high-density (5-10 units/ha)	High-density single family residential (>5 units/ha)
	Rural residential, high-density (>5 units/ha)
	Trailer parks and mobile home courts, high-density (>15 units/ha)
Single-family low-density (<5 units/ha)	Low-density single family residential
	Rural residential, low-density

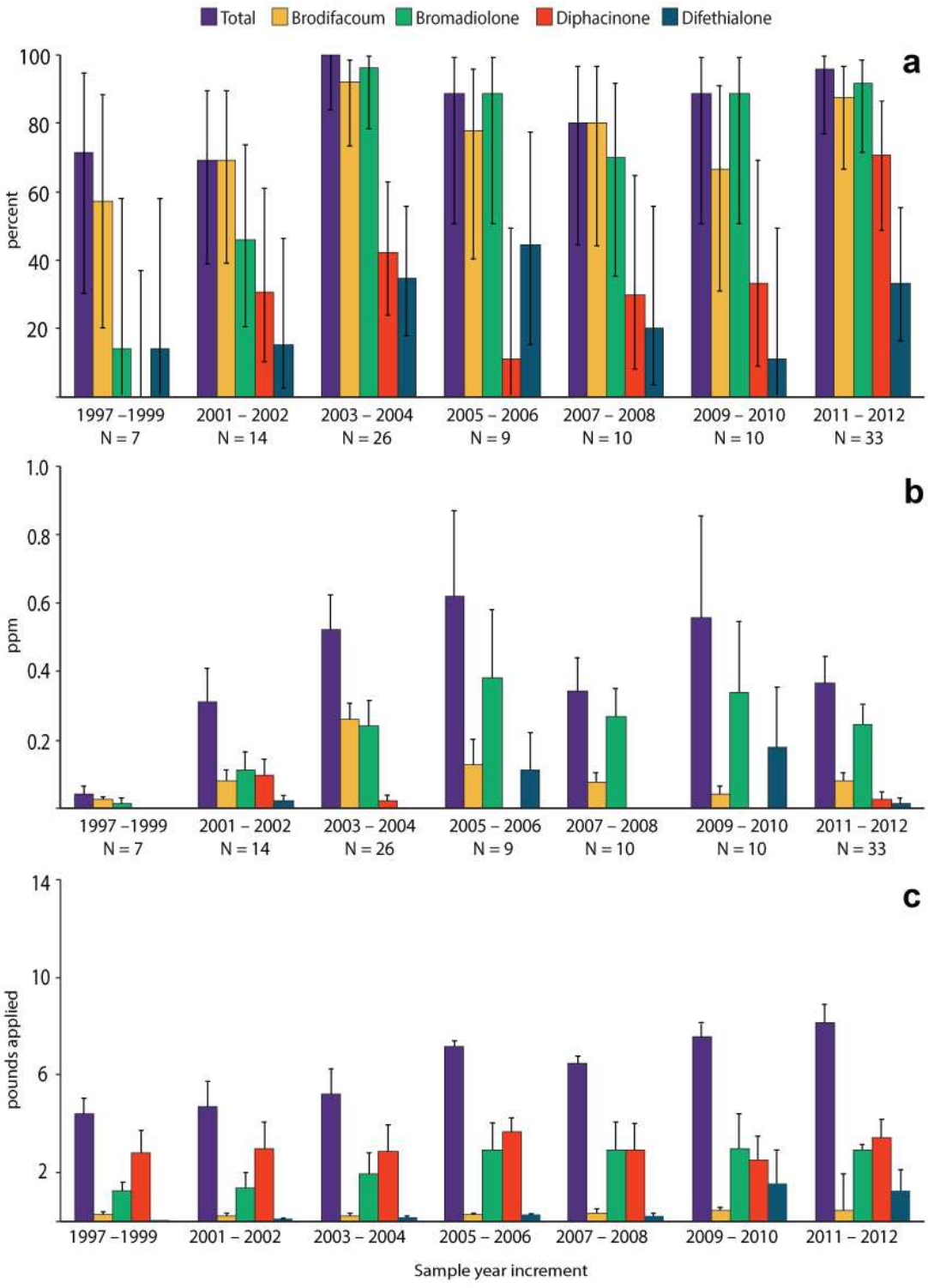

Figure B.1. AR data across 2-3 year time increments for the 4 most commonly detected compounds for SMMNRA only. a) Exposure prevalence overall and by compound per 2-3 year increment. Error bars represent 95% confidence intervals. b) Concentrations detected per 2-3 year increments. Error bars represent standard errors. c) Reported pounds of each compound applied per year increment in the 2 SMMNRA counties, Los Angeles and Ventura.

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CHAPTER 3

A pro-inflammatory response to sublethal toxicant exposure: exploring the causal link between anticoagulant rodenticides and severe notoedric mange in urban bobcat populations

ABSTRACT

Urbanization has profound ecological consequences and presents wild animal populations with a variety of novel stressors such as exposure to toxicants. The extent of sublethal physiological effects of toxicant exposure on wildlife is largely unknown. My primary objective was to examine the influence of sublethal, chronic exposure to anticoagulant rodenticides (ARs) on the health of bobcats in an urban landscape in southern California, by evaluating hematological, immunological, and serum chemistry parameters that are indicators of immune and organ function and nutritional status. An estimated 90% of individuals may be chronically exposed to ARs beginning during as early as prenatal development. Further, an epizootic of notoedric mange was the proximate cause of an extreme population decline from 2002-2005, and although there is a statistical link between mange and AR exposure, the causal link between the two was unknown. I sampled bobcats in both fragmented urban areas and natural protected areas in SMMNRA and performed complete blood counts, evaluated serum chemistries, and tested for AR exposure and for exposure or infection to a suite of common feline pathogens. I also adapted immunophenotyping assays to generate T and B lymphocyte profiles in bobcats. I robustly evaluated the effects of AR exposure on bobcat health by controlling for effects of demographic characteristics and status of infection with a variety of feline pathogens. Bobcats exposed to ARs exhibited immune stimulation, and specifically, polyclonal B cell proliferation, simultaneous to decreased percentages of neutrophils, indicative of immune suppression. I propose that polyclonal B cell proliferation, associated with AR exposure, supports the development of a specific type of immune response that contributes to uncontrolled mange infections. I also observed immunosenescence and B cell proliferation associated with feline immunodeficiency

virus exposure. These data highlight that even for free-ranging bobcats that may be resistant to direct AR toxicosis, sublethal consequences can increase their susceptibility to other stressors.

INTRODUCTION

Urbanization has profound ecological consequences, which can in turn result in a variety of novel stressors for wildlife populations. Habitat loss and fragmentation are obvious consequences of urbanization, while consequences such as exposure to toxicants (Riley et al. 2007) and increased disease susceptibility (Bradley and Altizer 2007) can have more cryptic effects on animal populations. Toxicants are a leading cause of species imperilment in the U.S. (Wilcove et al. 1998, Wilcove and Master 2005). Consequences of toxicant exposure can include direct mortality (Mendelssohn and Paz 1977, Aguilar and Borrell 1994, Riley et al. 2003, Gabriel et al. 2012), or sublethal, chronic effects such as reproductive impairment (McMurry et al. 1995, Vos et al. 2000, Eeva et al. 2003, Choi et al. 2004, Scheuhammer et al. 2007), and decreased immune competence (Choi et al. 2004). In natural populations, toxicants have also increase been associated with increased disease susceptibility and disease emergence (Ross 2002, Bradley and Altizer 2007, Riley et al. 2007).

Some toxicants are able to move through multiple trophic levels. One example of this type of secondary poisoning occurs in natural populations when a wide range of non-target animals, particularly predatory species, are affected by toxicants used to control rodent populations in urban and agricultural settings (Eason et al. 1996, Stone et al. 1999, Erickson and Urban 2004). Secondary poisoning is usually associated with compounds with delayed action such as first- and second-generation anticoagulant rodenticides (ARs) (Berny 2007). ARs are the most common form of chemical rodent control worldwide (Stone et al. 1999) and interfere with

the production of clotting factors produced by the liver, inducing mortality from hemorrhage (Erickson and Urban 2004). Second-generation ARs were developed to target rats with genetic resistance to first-generation ARs (FGAR) such as warfarin and in comparison with FGARs, they have considerably prolonged tissue persistence and increased potency (Petterino and Paolo 2001). Death from AR consumption can occur up to 10 days after ingestion (Cox and Smith 1992), meanwhile the target species may continue to accumulate the toxicants beyond the lethal dose. These prey species remain attractive to predators, however, and in becoming weakened by the poisons, they may become are easier to capture (Cox and Smith 1992, Berny et al. 1997). For predatory species that consume prey targeted by pesticides, chronic secondary exposure to the poisons may occur as a result, especially if use is widespread and constant.

Sublethal chronic exposure to ARs is suspected for multiple species of carnivores globally and is hypothesized to play an important role in epizootics of notoedric mange, an ectoparasitic disease in bobcat (*Lynx rufus*) populations in various parts of California (Riley et al. 2007, 2010, Serieys et al. 2013). During a long-term term bobcat study in Santa Monica Mountains National Recreation (SMMNRA) in southern California, a notoedric mange epizootic, strongly correlated with secondary AR exposure, was the greatest source of mortality for bobcats from 2002-2005 (Riley et al. 2007). During this period, the annual survival rate for radio-collared animals fell by >50% and in 2003, 51% of radio-collared animals died of mange. The disease epizootic had population-level impacts, significantly reducing the effective population size which caused to a genetic bottleneck (Serieys et al. in review, see Chapter 1). Susceptibility to severe mange in both the Santa Monica Mountains and elsewhere in California has been linked with secondary AR exposure (Riley et al. 2007, Serieys et al. 2013, Serieys et al. in review, see Chapter 2), although the mechanism by which ARs could increase susceptibility to

disease is unknown.

I investigated the influence of ARs on the physiological health of bobcats in SMMNRA, a diverse area that comprises both protected natural areas and highly fragmented urban areas. My primary objective was to examine the influence of AR exposure on the physiological and immunological health of bobcats, specifically on hematological, serum chemistry and immunological parameters, while controlling for the effects of demographic and seasonal factors and exposure to various feline pathogens. I measured AR exposure and exposure to or infection with feline panleukopenia virus (FPV), feline herpesvirus (FHV), feline calicivirus (FCV), feline immunodeficiency virus (FIV), puma lentivirus (PLV), Toxoplasma (*T. gondii*), and bacterial agents *Bartonella* sp. and *Mycoplasma* sp. I evaluated complete blood counts (CBC) and serum chemistry values. Further, I adapted immunophenotyping assays to characterize bobcat T- and B-lymphocyte profiles. To my knowledge, I am the first to immunophenotype, or identify and quantify specific lymphocyte populations, in bobcats. Finally, using long-term pathogen exposure and prevalence data, I measured the association between notoedric mange and coinfection with common feline pathogens that may also influence bobcat susceptibility to the ectoparasitic disease. These data offer an unprecedented opportunity to examine how disease, life history and toxicants may influence the immunological health of bobcat populations, a carnivore considered relatively adaptable to urban development (Crooks 2002).

I predicted that demographic and seasonal factors would influence hematological parameters in SMMNRA bobcat populations because studies conducted in a variety of taxa have shown that hematological values for wildlife may be influenced by age, sex, season, and nutritional and reproductive status (Fuller et al. 1985, Delgiudice et al. 1987), capture stress (Kocan et al. 1985, Marco and Lavín 1999, Serieys et al. 2013), and habitat quality (Seal and

Hoskinson 1978). I predicted that the prevalence of common feline pathogens would be low and would have minimal effect on hematological and immunological parameters because, with the exception of notoedric mange, clinical manifestations of disease have not previously been observed in the study area. However, I predicted that FIV infection may influence T cell profiles as has been previously described for domestic and wild cat species (Hoffmann-Fezer et al. 1996, Tompkins et al. 2002, Roelke et al. 2006). As found previously in a small-scale study, I also predicted that there would be no association between notoedric mange and coinfection with other feline pathogens (Serieys et al. 2013). Finally, I expected that AR exposure would be an important determinant of immunological profiles in individuals.

METHODS

Study Area

Bobcats were sampled in and around Santa Monica Mountains National Recreation Area (SMMNRA, Fig. 3.1a) in southern California from 1996-2014. The study area comprises more than 620 km^2, encompassing large regions of continuous habitat with minimal urban development, as well as highly fragmented areas that extend east through Los Angeles. The area straddles two major 8- to 10- lane freeways: US-route 101 and Interstate-405. Human development across the study area includes residential, commercial and agricultural development, as well as altered open areas such as golf courses and landscaped parks. Bobcat sampling occurred within highly fragmented habitat interspersed within urban areas (Fig. 3.1a, THOU and HLWD), highly fragmented habitat interspersed within an agricultural area (Fig. 3.1a, MOOR), and areas of moderate to no fragmentation (protected state and national park lands; Fig. 3.1a, MUGU, MALI, and TOPA).

Sample collection and processing

Bobcats were captured in cage traps (Tru-catch traps, Belle Fourche, South Dakota) or padded foothold traps checked every 12 hours as previously described (Riley et al. 2003, 2006, Serieys et al. 2013). A total of 229 samples were collected from 223 animals. All capture, handling, and sample collection protocols for individuals sampled from 2007-2012 were approved by the Office of Animal Research Oversight of the University of California, Los Angeles. Permits for sampling that occurred before 2007 or after 2012 were obtained by the National Park Service, and protocols were approved by California Department of Fish and Wildlife and National Park Service oversight committees. Once captured, bobcats were anesthetized with 5mg/kg ketamine HCl and 0.1mg/kg medetomidine and samples were collected in the field at the site of capture. Individuals were aged, sexed, weighed, measured, and ear-tagged. Blood samples were collected in both EDTA and serum separating tubes via jugular, cephalic, or saphenous venipuncture. Sedation was then reversed with HCl 0.5mg/kg atipamezole and individuals were released at the capture site.

Sample collection for complete blood counts, serum chemistries and immunophenotypes was initiated in 2007. Samples were intensively collected from 2009-2011 across the study area and 77% (N = 104) of the samples were collected during those two years. One-half milliliter of whole blood was used for complete blood counts. To obtain serum samples, blood was centrifuged within one hour of collection and serum was harvested. Serum chemistry and complete blood counts (CBC) were performed at a commercial laboratory (IDEXX, Irvine, California; N = 92) or the Division of Laboratory and Animal Medicine at University of California, Los Angeles, California (N = 30) within 24 hours of collection, with the exception of

5 individuals, whose blood chemistries were performed within one year of collection on serum frozen at -80 C.

Within 36 hours of blood collection at bobcat captures 1.5–15 ml of EDTA chelated blood samples, stored at 4^{o}C, were transported to the University of California, Los Angeles (UCLA) ImmunoBioSpot Core. To process blood for lymphocyte analyses, peripheral blood mononuclear cells (PBMCs) were purified using a Ficoll-Paque® (GE Healthcare Life Sciences, Pittsburgh, PA) histopaque protocol. Phosphate buffered saline (1xPBS) was added to the chelated blood after plasma removal such that there was approximately a 1:1 ratio of the starting volume of whole blood to PBS. The blood/PBS mixture was gently overlaid on 5 ml Ficoll-Paque®. The samples were immediately centrifuged for 25 min at 700 X G. The layer of PBMCs was then gently removed using a sterile transfer pipette, and added directly to 10 ml of 25^{o}C PBS. The PBMC/PBS mixture was centrifuged for 7 min at 300 X G. The supernatant was removed and cells were resuspended in 5 ml of PBS. Viable cells were quantified using trypan blue dead cell exclusion. The cell/PBS mixture was centrifuged for 7 min at 300 X G and the supernatant decanted. The cells were then resuspended in fetal calf serum (FCS) with 20% dimethyl sulfoxide (DMSO) yielding approximately 1 – 9 x10^{6} cells per 0.5 ml of the FCS/DMSO solution. One-half milliliter of the cell mixture was then quickly transferred to cryotubes containing 0.5 ml FCS. Each cryotube was placed in a Cyro Freezing Container (Thermo Scientific, Waltham, MA) and slowly frozen to -80^{o}C. After a minimum of 24 hours, samples were placed in liquid nitrogen until later thawing for flow cytometric analysis. Cryopreservation of the samples enabled cells collected on different dates to be viably preserved and assayed under standardized conditions simultaneously.

Immunophenotyping and flow cytometry

Within three minutes of removal from liquid nitrogen, cells were rapidly thawed in a 37^{o}C water bath. Next, 400 international units (IU) of DNAase (Sigma-Aldrich, St.Louis, MO, USA) was added directly to the 1 ml cell suspension and gently mixed using a pipette. Cells were slowly added to 10 ml of culture medium (15% FCS, 2% L-glutamine, 1% penicillin/streptomycin, 2.5% HEPES, and 81% RPMI) with 0.5 M EDTA, centrifuged at 300 X G for 6 min. and the supernatant was removed. Cells were next washed with PBS buffer with 1% BSA. The viability of cells was evaluated as described above, and only samples with a minimum viability of 70% (range 70-97%, mean = 85%) were retained for analysis. A minimum of 1 x 10^{6} cells were stained for immunophenotypic analysis. Forty microliters of cold heat-inactivated bobcat serum were added as a blocking agent, and cells were incubated for 20 min at 4^{o}C. Cells were labeled with one of three multicolor antibody panels to phenotype T and B lymphocytes and monocytes and macrophages: 1) CD5/CD4/CD8; 2) CD5/CD14/CD21; and 3) CD5/CD8/CD25. I used FITC-conjugated mouse anti-feline CD4 (clone 3-4F4 CD4; SouthernBiotech, Birmingham, AL, USA), PE-conjugated mouse anti-feline CD8 (clone fCD8; SouthernBiotech), biotin-conjugated mouse anti-feline CD5 (clone f43; SouthernBiotech), FITC-conjugated mouse anti-human CD21 (clone B-ly4; BD Biosciences, San Jose, CA, USA), FITC-conjugated mouse anti-feline CD25 (Tompkins Lab, North Carolina State University College of Veterinary Medicine, USA), and APC-conjugated mouse anti-human CD14 (clone Tük4; Invitrogen, Grand Island, NY, USA). Once antibodies were added, the cells were gently vortexed and incubated for 20 min at 4^{o}C. Thereafter, cells were washed with PBS buffer with 1% BSA. Streptavidin-PE/Cy7 solution (BD Biosciences) was used, per the manufacturer's instructions, to color label biotin-conjugated CD5-labeled cells. After cells were washed, they were resuspended

in 250 µl cold PBS buffer with 1% BSA and stored at 4^{o}C until flow cytometric analysis (within 3 hours). Twenty minutes prior to data acquisition, cells were labeled, per the manufacturer's instructions, with 7-AAD viability staining solution (BioLegend, San Diego, CA, USA) for dead cell exclusion. Unlabeled cells were used as negative controls and OneComp eBeads (BD Biosciences) were used for single-color compensation controls. The cells were acquired on a HT LSR II Flow Cytometer (BD). A minimum of100,000 events were collected during data acquisition by flow cytometric analysis for antibody-labeled cells while a minimum of 50,000 events were collected for unlabeled, negative controls.

Immunophenotypes were analyzed and quantified using FCS Express 4 (De Novo Software, Los Angeles, CA, USA). A dot-plot of side and forward scatter was used to construct a live lymphocyte gate. The percent of each cell type that included total T cells (CD5+), total B cells (CD21+), helper T cells (CD5+CD4+CD8-), cytotoxic T cells (CD5+CD4-CD8+), macrophages/monocytes (CD14+), and activated helper (CD5+CD25+CD8-) and cytotoxic T cells (CD5+CD25+CD8+) was determined for each sample. I also quantified CD5-CD4-CD8+ cells potentially representing natural killer cells (Vermeulen et al. 2012) and CD5+CD4+CD8+ cells. The absolute number of each cell population was calculated by multiplying the absolute number of lymphocytes per µl of whole blood that was determined during CBCs for each animal.

Tests for B cell clonality

I observed elevated B cell counts associated with anticoagulant rodenticide exposure. Consequently, I tested for B cell clonality to determine if B cells were derived from a single clone, as would occur in the case of chronic lymphocytic leukemia, or were polyclonal with varying antigen-binding regions, as would occur in reactive cell proliferation (Avery 2009). I

used two tests that included PCR for antigen receptor rearrangements (PARR) assays and serum immunofixation assays. While the use of both tests does not definitively indicate the clonality of B cells, they are the two methods available to test for monoclonality of B cells in domestic cats. Both procedures were performed at the Veterinary Diagnostic Laboratory at Colorado State University.

Serum immunofixation was performed on 0.5 ml of serum collected from a subset of 6 bobcats. Serum immunofixation is a standard semi-automated test using a Sebia HYDRASYS electrophoresis equipment (Norcross, GA, USA). Immunoglobulin G (IgG) proteins were separated by electrophoresis on alkaline buffered agarose gels, then incubated with individual specific antisera. After removing the non-reacted proteins, the immunoprecipitates were stained with acid violet. The electrophoregrams were evaluated for the presence of specific reactions with the sample proteins. Broad, diffuse smears in the IgG lane that corresponded with the light chain lane indicated the presence of polyclonal IgG response, suggestive of polyclonal B cells.

I additionally used the PARR assay on a subset of 7 bobcat whole blood samples as previously described (Burnett et al. 2003, Werner et al. 2005). Primers were developed for domestic cats and amplified T cell antigen genes (F: 5'- AAGAGCGAYGAGGGMGTGT -3', R: 5'-CTGAGCAGTGTGCCAGSACC -3') and Ig genes of B cells (DP17: 5'-CCGAGGACACGGCCACATATT-3', DP12: 5'-CTCTGAGGACACCGTCACCAG-3', and DP13: 5'-CTCTGAGGACACTGTGACTAT-3').

Pathogen surveys

Samples collected during 1996-2011 from 179 bobcats were tested for pathogen exposure (Table C.1, Appendix). All pathogen testing was carried out at the Center for Companion

Animals Studies at Colorado State University (CSU; Fort Collins, Colorado) or in the Feline Retrovirus Research Laboratory in the Microbiology, Immunology, and Pathology Department at CSU. Both whole blood and serum samples were used for disease testing, and samples were stored at -20^{o}C or -80^{o}C until testing. Serum samples were analyzed separately for FIV and PLV using western blot as previously described (Franklin et al. 2007a). Serum from blood samples was also assayed for FCV, FHV, *Bartonella* sp. and *T. gondii* specific antibodies by use of enzyme linked immunosorbant assay (ELISA) (Breitschwerdt et al. 1995, Lappin et al. 2002, Luria et al. 2004, Vollaire et al. 2005, Lappin et al. 2006). In all cases serological tests were against IgG antibodies. To test for *M. haemofelis, M. haemominutum, B. henselae* and *B. clarridgeaie* infection, specific PCR assays were performed on whole blood (Jensen et al. 2001, Barrs et al. 2010). Finally, to evaluate mange infection, all animals were visually inspected for clinical signs of mange that included severe dermatitis, alopecia, and lichenification of the skin. If clinical mange was observed, skin scrapings in the affected areas were performed to confirm mite species (Riley et al. 2007, Serieys et al. 2013, Stephenson et al. 2013).

Anticoagulant rodenticide (AR) screening

Residues, both presence and amount, of warfarin, coumachlor, bromadiolone, brodifacoum, diphacinone, chlorophacinone, and difethialone were assessed in whole blood (2 ml) or serum (1 ml) by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS/MS) as previously described (Waddell et al. 2013). Limits of quantification was 1 parts per billion (ppb) for each compound with method detection limits ranging from 0.28 to 0.45 ppb. ARs that were determined to be positive by LC-MS/MS, but were below the limit of quantitation by HPLC, were defined as above the limit of detection

(LOD) or "above LOD". In one case, probable AR exposure was assessed using a test of proteins invoked in the absence of vitamin K (PIVKA, Serieys and Riley, unpubl. data) as previously described (Whisson and Salmon 2002) and residue concentration data was unavailable for this individual.

Natural land use

I used the percent of natural area in a circular polygon (buffer zone) drawn around each bobcat capture location as a second metric of AR exposure.M reasoning for the use of this second metric is two-fold. First, I found during previous studies that the use of blood for AR exposure detection detects less than half of exposure detected using liver tissue, and thus the blood test alone likely does not capture the true exposure prevalence in my populations. Second, I also found that AR exposure was negatively associated with the percent of natural area in bobcat buffer zones (Serieys et al. in review, see Chapter 2). Thus, the use of two metrics of AR exposure more robustly tests the influence of AR exposure on bobcat health parameters if statistical analyses reveal concordant results for both metrics. To evaluate the surrounding landscape of all captured bobcats, I created circular buffer zones with capture location as the centroid as previously described (Serieys et al. in review, see Chapter 2). Female buffer zones (2.3 km^2) and male zones (5.2 km^2) were equal to the size of a 95% minimum convex polygon home range for that sex at SMMNRA (Riley et al. 2010). I clipped the 2005 land use dataset provided by Southern California Association of Governments (SCAG, http://gisdata.scag.ca.gov/Pages/Home.aspx) with bobcat buffer zones in ArcGIS 10.2 (ESRI, Redlands, CA) to quantify the land types used by each bobcat. Within the circular polygon, I

quantified the total percent of undeveloped natural areas that included state and national parks, other undeveloped open space and recreational areas and regional parks.

Body condition residuals

As a measure of body condition, I calculated the residuals from an ordinary least square (OLS) linear regression of body mass plotted against body length. The relationship between body mass and body length has been validated as a body condition measure in a variety of vertebrate taxa including carnivores (Cavallini 1996, Green 2001, Brock et al. 2013, Labocha et al. 2014). Variation in mass per unit length is most often calculated for adult animals, and it is assumed that higher values are indicative of better nutritional condition. Because sex (Cavallini 1996) or age (Brock et al. 2013) may influence the mass/length relationship, the body condition index for animals in this study were calculated separately for adult males, adult females, juvenile males and juvenile females. I used body weight and length measurements collected in the study area since 1996 as reference values (Riley et al. NPS unpubl.data) with which to plot the regression and calculate the residuals. A total of 93 body residuals were available for analyses.

Statistical analyses

Descriptive statistics for blood chemistries, CBCs and immunophenotypes are presented as mean +/- standard error and median. Prevalence and exact 95% confidence intervals of AR and pathogen exposure or infection were calculated. For pathogen prevalence estimates, in the case of recaptures, I used only the most recent capture event to estimate prevalence.

We evaluated the influence of AR exposure, percent natural areas within individual buffer zones, sex (male, female), season sampled (wet: November 1 – April 30, dry: May 1 –

October 31), age class (adult animals ≥ 2, juveniles < 2 years, kitten < 3 months), and exposure to or infection with FIV, *T. gondii*, and *Bartonella* sp. or infection with *B. henselae*, *M. haemominutum* on immunophenotypes, CBCs, serum chemistry values, and body condition. Because only 2 kittens were sampled for serum chemistry analyses, they were excluded from blood chemistry analyses. Exposure to FCV, FHV, PLV or infection with *B. clarridgeiae* and *M. haemofelis/turicensis* were also excluded from statistical analyses because their prevalence was low (<10%, Fig. 2.2, Table C.1, Appendix) and so I lacked sufficient statistical power to evaluate the effect of these pathogens on the health of bobcats. Analyses were performed using two types of regression models: OLS or generalized linear models (GLM) with a log link. The use of each type of model was determined according to the distribution of each set of health parameter data evaluated with histograms of the distribution of the data for each health parameter. Because of the special sample handling requirements, physiological data was available beginning only in 2007. Thus, regression analyses were performed on a dataset of 120 individuals for which complete blood counts, blood chemistries, or immunophenotypes were available. Of these 120 samples, 74 were collected from adults, 38 from juveniles, and 6 from kittens (< 3 months). Fifty animals were female and 68 were male (Table 3.1).

Next, to test the influence of independent variables on the dependent parameter while controlling for all significant independent variables, I performed multivariate regression models, using OLS regressions or GLMs depending on the distribution of the data as described above. The multivariate regression models were selected by backward stepwise selection using Akaike's Information Criterion (AIC) for model selection. I selected and report the strongest models with ΔAIC values ≤ 2 (Burnham and Anderson 2002). For all regression analyses, I report the odds ratio, interpreted as how the odds of the health parameters change as a function of the predictor

variables. I also report the 95% confidence interval of the odds ratio, and the model *P*-values. I calculated odds ratios and the 95% confidence intervals of the odds ratio by exponentiating each model's coefficients and confidence intervals.

When evaluating the effect of AR exposure, life history, season, and all pathogens with the exception of *N. cati* on health parameters, I used only data collected from individuals that showed no clinical signs of mange because previously, I documented significant CBC and serum chemistry anomalies in bobcats with clinical mange (Serieys et al. 2013). For physiological (CBC, blood chemistry, and immunology) data analyses, recaptured animals were counted only once, and only the sampling date with most complete dataset (pathogen/AR exposure information) was used. Two recaptured animals had incomplete datasets for both capture events, and between each capture event there were no overlapping data on pathogen/AR exposure, blood chemistries, CBCs, and immunophenotypes. Thus, for these two animals, I used information collected on both capture events but used life history (sex, age class) and seasonal information for these two animals only from the most recent capture event.

Finally, I used Fisher's exact tests to examine potential associations between severe clinical mange and coinfection with FIV, FCV, FHV, *Bartonella* sp., *T. gondii, M. haemominutum* or *B. henselae.* These analyses were performed using the pathogen prevalence data collected from 1996-2011. I also used Fisher's exact tests to test for nonrandom patterns of coinfection between viral, bacterial and *T.gondii* with the 2007-2011 dataset because coinfection between viral and bacterial pathogens has been found to influence the susceptibility and physiological response of domestic cats to the bacterial pathogens, especially *Mycoplasma* sp. (Sykes 2010, Sykes et al. 2010). All statistical analyses were performed in R (R Development Core Team, 2012). I did not correct for multiple tests because methods of correction for multiple

tests tend to have a higher probability of generating Type II errors in comparison with Type I errors (Moran 2003). Tests were considered significant when $\alpha \leq 0.05$.

RESULTS

Anticoagulant rodenticides

Anticoagulant rodenticide exposure data were available for 92 animals. Exposure prevalence to any AR compound was 41% (N = 38, 95% CI: 31–52%). Bobcats were exposed to up to 4 different compounds, although for 87% of the 37 bobcats, I detected one AR compound. For three individuals, two compounds were detected, for one individual three compounds were detected, and for one individual, 4 compounds were detected. Of the 37 positive bobcats, 33 (89%) were exposed to the first-generation ARs diphacinone, 4 to chlorophacinone, and two to coumachlor and 4 were exposed to the second-generation ARs brodifacoum and three to bromadiolone. ARs were most frequently detected at above LOD levels, quantifiable only in 9 instances (20% of all detections). I was thus unable to determine potential dose effects of ARs on the health parameters measured in this study.

Anticoagulant rodenticide exposure was a significant predictor for white blood cell counts (Fig. 3a-b, Table 3.2), immunophenotypes (Fig. 3.3c-d, Table 3.3, Table C.2, Appendix), and clinical chemistry values (Table 3.4). Total lymphocytes were elevated on average by 42% in exposed bobcats ($P = 0.02$, OR = 1.42, 95% CI: 1.07–1.93; Fig. 3.3a), and specifically, the percentage of B-lymphocytes was elevated by 36% in exposed bobcats ($P = 0.03$, OR = 1.35, 95% CI: 1.04–1.78)(Fig. 3.3c). The average absolute number of B lymphocytes in positive bobcats was nearly twice as high in negative bobcats ($P = 0.004$, OR = 2.07, 95% CI: 1.33–3.62; Fig. 3.3c). B cell counts and percentages were highest in populations with greater AR detection

prevalence (Fig. 3.1b). With regard to measures of innate immunity, the percentage of neutrophils was 9% lower in positive bobcats (P = 0.031, OR = 0.002, 95% CI: 0.0001–0.05; Fig. 3.2b). Total bilirubin was 26% lower (*P* = 0.03, OR = 0.80, 95% CI: 0.64–0.98) in bobcats with detectable ARs (positive: mean = 0.22 mg/dl, SE = 0.66, median = 0.30; negative: mean = 0.28 mg/dl, SE = 0.02, median = 0.20). Phosphorus was 10% higher (*P* = 0.029, OR = 1.74, 95% CI: 2.85) in exposed bobcats (positive: mean = 6.38 mg/dl, SE = 0.18, median = 6.35; negative: mean = 5.82 mg/dl, SE = 0.17, median = 5.90).

As with AR exposure, the percent natural area in bobcat buffer zones was a significant predictor for immunophenotype and clinical chemistry values. I found a negative association between the percent of natural area and the absolute number (*P* = 0.011, OR = 0.43, 95% CI: 0.23–0.82) and the percent of B lymphocytes (*P* = 0.001, OR = 0.52, 95% CI: 0.36–0.77; Table 3.2). Because anticoagulant exposure is negatively associated with the percent of natural area in buffer zones (Serieys et al., in review, see Chapter 2), these results support the association between AR exposure and B cell expansion (elevated B cell counts). I observed a positive association between the percent of double-positive CD4+CD8+ T cells and the percent natural area in buffer zones (*P* = 0.021, OR = 5.00, 95% CI: 1.55–33.45; Table 3.2), although the biological significance of this type of immune activation is unknown. Finally, I found a positive association between total bilirubin and the percent of natural area (*P* = 0.010, OR = 1.12, 95% CI: 1.09–1.23; Table 3.4) and a negative association between percent of natural area and serum phosphorus (*P* < 0.001, OR = 0.19, 95% CI: 0.08–0.43; Table 3.4).

B cell clonality

I used two methods to test whether B cell expansion resulting from AR exposure was monoclonal or polyclonal. Using PARR testing designed for domestic cats, I attempted to sequence immunoglobulin genes, although the primers did not work for the bobcats tested, and thus those results are inconclusive. However, I also performed serum immunofixation, and IgG tests were performed on 6 bobcats, 5 of which were exposed to ARs and had among the highest B cell counts. All animals showed the presence of polyclonal IgG response. Thus, using the tools available, B cell proliferation associated with AR exposure appears to be polyclonal.

Pathogens

Seroprevalences were similar across the 1996-2011 dataset compared with the 2007-2011 subset of animals for which both disease and physiologic data was available (Fig. 3.2, Table C.1, Appendix). Individuals were most frequently infected with *B. henselae* (prevalence: $\geq$ 60%) and *M. haemominutum* (prevalence: $\geq$ 47%), while among viral pathogen exposure, FIV was most frequently detected (prevalence: $\geq$ 20%, Fig. 3.2). From 1996-2011, I documented 23% (95% CI: 0.17 – 0.30) of 200 bobcats sampled in my study area to be afflicted with notoedric mange, either at the time of capture (N = 7), or to later develop the disease (N = 39). Using the disease prevalence data collected from 1996-2011, I found no association between notoedric mange and exposure to FIV (P = 0.83), FCV (P = 0.35), FHV (P = 0.55), *Bartonella* sp. (P = 0.71), or *T. gondii* (P = 0.67) or to infection with *M. haemominutum* (P = 0.17) or *B. henselae* (P = 0.36). Using the 2007-2011 dataset, I observed a coinfection association between *M. haemominutum* and *T. gondii* (P = 0.02, OR = 5.95, 95% CI = 1.09–61.16), although coinfection with these two

pathogens has not been documented to increase the severity of clinical manifestations of disease for either pathogen in domestic cats.

FIV and *M. haemominutum* were the two most important pathogen-related predictors of health parameters (Table C.3, Appendix). As with AR exposure, FIV exposure was associated with a pro-inflammatory B lymphocyte response. FIV seropositive bobcats had 67% more (P = 0.047, OR = 1.65, 95% CI: 1.00–2.77; Table 3.2) B lymphocytes per µl (positive: mean = 520.9, SE = 11.6, median = 447.9; negative: mean = 314.4, SE = 48.4, median = 261.9). The mean percentage of total B lymphocytes was 45% greater (P = 0.022, OR = 1.45, 95% CI: 1.05–1.98) in FIV seropositive bobcats (positive: mean = 29.7%, SE = 1.8, median = 29.1; negative: mean = 20.5%, SE = 4.5, median = 20.6). Bobcats exposed to FIV also had 73% higher (P = 0.002, OR = 1.73, 95% CI: 1.25–2.42) percentage of CD5+ B-lymphocytes (positive: mean = 0.57%, SE = 0.1, median = 0.41; negative: mean = 0.33%, SE = 0.03, median = 0.33).

M. haemominutum infection was also associated with multiple health parameters including immunophenotypes, CBCs, and blood chemistries (Table C.3, Appendix). Of particular interest and in contrast with the B cell pro-inflammatory response to FIV exposure, bobcats infected with *M. haemominutum* had approximately 50% fewer B lymphocytes (infected: mean = 280.4 cells/µl, SE = 35.4, median = 259.4; uninfected: mean = 595.7 cells/µl, SE = 86.6, median = 413.0; P = 0.011, OR = 0.47, 95% CI: 0.23–0.77; Table 3.2). Potential immune response to the pathogen was also evident in complete blood counts (Table C.3, Appendix). Infected bobcats had, on average, 19% higher (P = 0.014, OR = 1.19, 95% CI: 1.04–1.37; Table 3.3) leukocyte counts (infected: mean = 14.9 K/µl, SE = 0.8, median = 14.4; uninfected: mean = 12.5 K/µl, SE = 0.6, median = 11.7). However, the average percentage of lymphocytes was approximately 36% lower (P = 0.028, OR = 0.73, 95% CI: 0.55–0.95) in infected bobcats

(infected: mean = 12.3%, SE = 1.4, median = 14.4; uninfected: mean = 16.7%, SE = 1.3, median = 16.5). Neutrophil counts were 25% greater (P = 0.023, OR > 10,000, 95% CI > 10,000) in infected bobcats (infected: mean = 11.6 K/μl, SE = 0.8, median = 10.6; uninfected: mean = 9.3 K/μl, SE = 0.6, median = 9.3). Finally, infected bobcats (mean = 0.5 K/μl, SE = 0.04, median = 0.5) had an average of 29% more monocytes (P = 0.023, OR > 10,000, 95% CI > 10,000) than uninfected bobcats (mean = 0.4 K/μl, SE = 0.02, median = 0.4).

Age class

Age class significantly influenced immunophenotype and hematological parameters. Specifically, I observed evidence of age-driven immunological remodeling and immunosenecence (Table 3.5). The percentage of helper T cells was 56% lower in adult bobcats (Table 3.5) compared with juveniles (P = 0.005, OR = 1.32, 95% CI: 1.09 – 1.59; Table 3.2) and although only weakly significant, adult bobcats had an average of 46% fewer absolute helper T cells compared with juvenile bobcats (P = 0.07, OR = 1.46, 95% CI: 0.96 – 2.21). Juveniles had 50% higher percentages of CD5+ B cells (P = 0.039, OR = 1.35, 95% CI: 1.01 – 1.80). The absolute number of total leukocytes (Table 3.5) in adults was 17% higher than in juveniles (P = 0.029, OR = 0.86, 95% CI: 0.74–0.98; Table 3.3). However, both the absolute number (P < 0.001, OR = 2.48, 95% CI: 1.81–3.30; Table 3.3) and percentage (P < 0.001, OR = 3.39, 95% CI: 2.63–4.35) of lymphocytes was more than twice as high in kittens compared with adults and juveniles (Table 3.5). The absolute number (P = 0.005, OR > 10,000, 95% CI: > 10,000) and percentage (P < 0.001, OR <0.00001, 95% CI: <0.00001) of neutrophils was significantly lower in kittens compared with adults and juveniles (Table 3.5).

Age class also significantly influenced numerous clinical chemistry values (Table 3.5). Changes included juveniles having 34% higher serum alkaline phosphatase ($P < 0.001$, OR = 3.34, 95% CI: 2.13–6.45), 5% higher calcium ($P < 0.001$, OR = 1.05, 95% CI: 1.03–1.07), 19% higher phosphorus ($P < 0.001$, OR = 2.98, 95% CI: 1.96–4.52), and 58% higher creatinine ($P < 0.001$, OR = 0.63, 95% CI: 0.52–0.75).

Sex

Sex was a significant predictor of white blood cell counts and blood chemistry parameters (Table C.4, Appendix). For example, males had 14% higher ($P = 0.023$, OR = 1.16, 95% CI: 1.02 – 1.32) mean leukocyte counts than females (male: mean = 14.3 K/µl, SE = 0.6, median = 13.1; female: mean = 14.3 K/µl, SE = 0.6, median = 13.1).. Males also had multiple significantly elevated red blood cell values in comparison with females (Table C.4, Appendix). Red blood cell counts were 6% greater ($P = 0.029$, OR = 1.53, 95% CI: 1.05–2.24; Table 3.3), hematocrit was 6% greater ($P = 0.023$, OR = 1.06, 95% CI: 1.01–1.12), and hemoglobin was 4% greater ($P = 0.047$, OR = 1.72, 95% CI: 1.01–2.95) in males. Additionally, creatinine was 18% higher ($P = 0.046$, OR = 1.18, 95% CI: 1.01–1.39) in males than in females (male: mean = 1.7 mg/dl, SE = 0.1, median = 1.6; female: mean = 1.5 mg/dl, SE = 0.1, median = 1.5), likely reflecting differences in muscle mass between the sexes.

Season

I observed seasonal differences in body condition, red blood cell counts, and several serum chemistry values. Body condition was significantly greater ($P = 0.006$, OR = 1.60, 95% CI = 1.15 – 2.22) in the wet season compared with the dry season (wet: mean= 0.04, SE = 0.14,

median = -0.02; dry: mean = -0.43, SE = 0.08, median = -0.44). Red blood cell counts, hemoglobin, and hematocrit were lower during the wet season. During the dry season, the mean red blood cell count value was, on average, 7% higher (dry: mean = 8.15 M/μl, SE = 0.12, median = 8.05; wet: mean = 7.62 M/μl, SE = 0.16, median = 7.77). Hematocrit values were elevated by an average of 9% during the dry season (dry: mean = 40.81%, SE = 0.61, median = 40.10; wet: mean = 37.31%, SE = 0.77, median = 36.40). Hemoglobin values were, on average, 5% higher during the dry season (mean = 12.89 g/dl, SE = 0.17, median = 12.70; wet: mean = 12.26 g/dl, SE = 0.21, median = 12.30), while the mean corpuscular hemoglobin concentration was on average, 4% higher in the wet season (wet: mean = 33.11 g/dl, SE = 0.40, median = 33.00; dry: mean = 31.69 g/dl, SE = 0.27, median = 31.90). Serum aspartate aminotransferase (AST) levels were an average of 28% higher during the wet season (wet: mean = 115.14 U/l, SE = 9.98, median = 95.00; dry: mean = 82.87 U/l, SE = 5.94, median = 66.00). Total protein values were an average of 3% higher during the dry season (dry: mean = 7.24 g/dl, SE = 0.07, median = 7.2; wet: mean = 7.06 g/dl, SE = 0.05, median = 7.05), while globulin values were an average of 7% higher during the dry season (dry: mean = 3.82 g/dl, SE = 0.09, median = 3.80; wet: mean = 3.58 g/dl, SE = 0.07, median = 3.50).

Multivariate models

A total of 9 multivariate models were found to best-explain various health measures used in this study (Table 3.6). Of particular importance, exposure to ARs (P = 0.027, OR = 1.74, 95% CI: 1.02–2.63) and FIV exposure (P = 0.039, OR = 1.64, 95% CI: 1.10–2.98) were the best predictors of absolute B lymphocyte counts. Total leukocyte counts were best predicted by sex (P = 0.011, OR = 1.64, 95% CI: 1.10–2.98) and *M. haemominutum* infection (P = 0.009, OR =

1.20, 95% CI: 1.05–1.37) while absolute lymphocyte counts were best explained by age class ($P < 0.001$, OR = 3.40, 95% CI: 1.67–5.62) and exposure to ARs ($P = 0.008$, OR = 1.51, 95% CI: 1.12–2.07). Infection with *M. haemominutum* ($P = 0.025$, OR = 471.34, 95% CI: 2.40–9,273.37) and exposure to ARs ($P = 0.011$, OR = 0.009, 95% CI: <0.001–0.18) were the best predictors of the percent of neutrophils. Thus, even after controlling for the immune response to various other pathogens, AR exposure was an important predictor of absolute B cell counts, lymphocyte counts, and the percentage of neutrophils.

DISCUSSION

Anticoagulant rodenticide exposure influence on health parameters

Anticoagulants are vitamin K antagonists which interrupt production of vitamin-K dependent blood clotting proteins (Shearer 1990). During a recent study, I used liver and blood samples to evaluate AR exposure from 1996-2012 in southern California (see Chapter 2). Using liver tissue, I detected approximately 90% exposure in 169 individuals across my study area, the four most commonly detected compounds were second-generation compounds brodifacoum, bromadiolone, and difethialone, and the first-generation compound diphacinone. Using blood samples, 39% of 195 individuals were exposed, and I most frequently detected diphacinone. Anticoagulant exposure detected in blood likely reflects only recent exposure events; however, animals in my study area may experience sublethal, chronic exposure to ARs that can begin during prenatal development and likely extend for the duration of an individual's life (Serieys et al. in review, see Chapter 2).

Domestic cats appear more tolerant to the anticoagulant effects of rodenticides in comparison with a variety of other taxa (Erickson and Urban 2004), and this tolerance may also

be experienced by bobcats. With rare exception I have not found evidence of clinical AR toxicosis in the study area despite widespread exposure to both first- and second-generation ARs (Riley et al. 2010, Serieys et al. 2013, Serieys et al. in review). However, chronic AR exposure has been hypothesized to increase bobcat susceptibility to notoedric mange, an ectoparasitic disease (Riley et al. 2007, Serieys et al. 2013, Serieys et al. in review), although until now, I have not known the potential immune effects of AR exposure. Further, there are few reports of a potential interaction between infectious disease and AR exposure. In addition to bobcats, notoedric mange was also noted in 2 mountain lions exposed to ARs and Riley et al. (2007) hypothesized that AR exposure also increases mountain lion susceptibility to severe mange. A possible interaction between the toxic effects of chlorophacinone, a first-generation AR, and a bacterial pathogen, *Francisella tularensis* that causes tularemia in common voles (*Microtus arvalis*) has also been reported (Vidal et al. 2009). Voles that were infected with *F. tularensis* required lower lethal doses of chlorophacinone than uninfected voles. Tularemia prevalence was also higher in areas treated with chlorophacinone, and the authors suggested that the AR field treatment may have also facilitated the spread of the disease in the affected vole population.

Warfarin, a first-generation AR (FGAR), has been widely used therapeutically to prevent blood-clotting disease in humans, and thus the physiological effects, particularly with respect to Vitamin K antagonism, of warfarin have been extensively studied in humans and rats (Popov et al. 2013). However, any potential effects of the drug on the immune system are the less understood (Belij et al. 2012). Second-generation ARs (SGARs) brodifacoum and bromadiolone, like warfarin, are 4-hydroxycoumarin (coumarin) derivatives (Felice et al. 1991). Beyond anticoagulant properties, no studies describe the physiological effects of SGARs; however, as coumarin derivatives I expect that any potential immunological effects they may have would be

similar to if not more severe than those of warfarin given the enhanced toxicity and prolonged tissue persistence of SGARs.

Coumarin derivatives have both immunostimulatory (Berkarda et al. 1983, Belij et al. 2012) and immunosuppressive properties (Perez et al. 1994, Kurohara et al. 2008, Belij et al. 2012). The immune effects of coumarin derivatives are suggested to be dose dependent. In low, chronic doses, warfarin may be immunosuppressive, but it may be pro-inflammatory at higher doses (Kater et al. 2002). However, a recent study noted simultaneous immunostimulatory and immunosuppressive effects (Kater et al. 2002, Popov et al. 2013). Using both metrics of AR exposure including residue detection in blood and the percent natural area in buffer zones, I found strong evidence of immunostimulatory polyclonal B cell expansion in response to AR exposure. Although the PARR test to evaluate B cell clonality did not function in the bobcats, clonality testing using both methods performed on mountain lion samples showed polyclonal B cells in AR exposed mountain lions (Serieys et al. unpublished data). Because it has been hypothesized that the relationship between ARs and mange are the same for mountain lions (Riley et al. 2007), it may be that the results of mountain lion B cell clonality testing support similar polyclonal B cell proliferation in bobcats. Polyclonal B cell expansion rules out the possibility of neoplasia, or lymphocytic leukemia, in the bobcats as a result of AR exposure. Finally, I also observed lower percentages of neutrophil granulocytes in AR-exposed bobcats, suggestive of a simultaneous immunosuppressive response to AR exposure.

To my knowledge, there are no studies that have investigated the effects of coumarin derivatives on B lymphocytes. However, coumarin derivatives were first reported to have immunostimulatory properties on T lymphocytes more than 30 years ago. Elevated T lymphocyte counts and enhanced mitogen-stimulated lymphocyte proliferation occurred in

humans after only 8 days of receiving a daily therapeutic dose of warfarin or coumarin (Berkarda et al. 1983). More recently, Popov et al. (2013) observed both immunosuppressive and immunostimulatory effects of warfarin in laboratory rats. Warfarin caused a significant decrease in granulocytes and a decrease in granulocyte production of tumor necrosis factor alpha (TNFα), a cytokine important in the regulation of immune-related cells, simultaneous with an increase in lymphocytes and monocytes. The decrease in granulocyte TNFα expression could contribute to immune suppression in rats chronically dosed with warfarin (Popov et al. 2013). In contrast, when peripheral blood mononuclear cells (PBMC) from rats treated with warfarin for 30-days were challenged by lipopolysaccharides, they produced elevated levels of IL-6, a pro-inflammatory cytokine. In addition, PBMC in warfarin-treated rats also have increased oxidative activity (Belij et al. 2012), and collectively, the increased oxidative activity and increased PBMC IL-6 production is evidence of the pro-inflammatory effects of subchronic oral intake of the coumarin derivative (Popov et al. 2013). Interestingly, IL-6 activity is closely linked with B cell growth and differentiation (Murphy 2012). Thus, chronic ingestion of the toxicants that are coumarin-derivatives may similarly cause increased IL-6 activity in bobcats, leading to B cell proliferation in individuals.

The potential interactive effects that diphacinone may have with coumarin derivatives is of particular concern. The FGAR diphacinone was the agent most frequently detected in bobcat blood. Diphacinone differs from may of the SGARs in that it is an indandione derivative, although utilized clinically in similar situations as coumarins (Naisbitt et al. 2005). However, indandione derivatives are known to induce an immunoallergenic dermal reaction, lymphocyte proliferation (Naisbitt et al. 2005) and neutropenia (Perkins 1962). These findings underscore

concerns for direct and interactive effects on immune or other physiological function in nontarget species exposed to the toxicants.

Pathogens and disease

I observed bobcat exposure to multiple feline pathogens. Although numerous studies have evaluated pathogen exposure prevalence in wild felid populations (Heidt et al. 1988, Roelke et al. 1993, Daniels et al. 1999, Labelle et al. 2001, Riley et al. 2004, Roelke et al. 2007, Bevins et al. 2012, Foley et al. 2013), and specifically in bobcats (Heidt et al. 1988, Labelle et al. 2001, Riley et al. 2004, Franklin et al. 2007b), little is known about clinical importance of these diseases for wild felids. The rates of infection with PLV, FCV, and FHV were low, and so I was unable to determine potential physiologic impacts these diseases may have on infected bobcats. Although I found associations between physiological parameters and FIV, *Mycoplasma*, *Bartonella*, and *T. gondii*, health parameters associated with these pathogens did not fall outside of reference hematological or serum chemistry value intervals for domestic cats, and so I have no evidence that these diseases are of clinical importance for these bobcat populations. Importantly, I found no association between notoedric mange and coinfection with other disease, suggesting that if immune-related anomalies are associated with increased susceptibility to notoedric mange, these anomalies are not caused by coinfection, nor do they give rise to higher coinfection, with the pathogens I investigated.

Most interesting, and similar to AR exposure, FIV was associated with a pro-inflammatory B cell response. For FIV-infected domestic cats, clinical signs of infection are typically nonspecific and unobserved, except in the case of opportunistic secondary infection with other pathogens (Gleich and Hartmann 2009). FIV typically targets lymphocytes and

macrophages in domestic cats leading to a gradual loss in helper T cell numbers and function, immune deficiency, and increased susceptibility to other pathogens (Tompkins et al. 2002). However, in domestic cats, infection is also characterized by polyclonal expansion of B cells, as I observed in the bobcats (Flynn et al. 1994, Hartmann 1998, 2012).

Chronic polyclonal B cell expansion and susceptibility to severe mange

Chronic polyclonal B cell expansion may play an important role in bobcat increased susceptibility to mange. For example, chronic polyclonal B cell activation has been suggested to lead to B cell exhaustion which could contribute to immune suppression in FIV-infected cats (Flynn et al. 1994). If AR exposure leads to elevation of B cells, immune exhaustion could similarly occur in individuals chronically exposed to ARs. Alternatively, polyclonal B cell activation and proliferation, without pathogen specificity, may enable microorganisms to evade a host-specific immune response (Montes et al. 2007). In both humans and mice, B cells also play a critical role in autoimmune disease, which can be exacerbated when stimulated by pathogens or drugs (Granholm and Cavallo 1992). Potentially, infection with mange could be one such stimulus that could lead to the development of autoimmune disease which could complicate the ability of bobcats to mount an effective anti-mite immune response. Overall, with each of these potential scenarios, I would expect generalized susceptibility to any pathogen, and within the study area there is no evidence that AR exposure has increased bobcat susceptibility to diseases other than mange. However, recent research has pointed to important roles that B cells may play in allergic responses (Samitas et al. 2010) that may selectively increase bobcat susceptibility to severe mange (see below).

To my knowledge, there are no studies that describe the important immune pathways in wildlife populations, particularly in response to notoedric mange. However, for human response to the closely related scabies mite, *Sarcoptes scabiei*, an immune response dominated by a Th1 cell-mediated protective response, rather than a Th2 allergic response, helps the patient control the parasite (Walton et al. 2008, Walton 2010). The Th1 type immune response is dominated by CD4+ (helper T) that secrete cytokines IFN-γ and IL-2. In contrast, the Th2 response is characterized by helper T cells that secrete cytokines IL-4, IL-5, and IL-13 and leads to IgE-mediated hypersensitive allergic reaction (Murphy 2012).

The increasingly recognized characteristics of B cells and their involvement with allergic disease (Samitas et al. 2010) may best explain the link between AR exposure, chronic B cell expansion, and severe mange parasitism in bobcats. The mechanisms responsible for determining the balance of Th1/Th2 response are poorly understood, although B cells may play a role in the development of Th2 dominated hypersensitive responses to environmental stimuli (Lindell et al. 2008). For example, a recent study found that B cells presenting antigen to T cells could affect the nature of the cytokine response, and thus determine the skew of a Th1/Th2 response. When mouse T cells were co-cultured with pulmonary B cells presenting cockroach-antigen, the T cells produced high levels of Th2 cytokines but very little Th1 cytokines (Lindell et al. 2008). Therefore, chronic B cell expansion in bobcats may lead to B cell presentation of mange antigen to T cells, stimulating a Th2 dominated response to mange infection. This scenario may best explain the extreme mange parasitism, and widespread prevalence of the disease, in chronically AR exposed bobcat populations. Most importantly, the relationship between B cells and the initiation of a Th2 dominated allergic response would explain why bobcats are specifically susceptible to mange, rather than having susceptibility to pathogens in general. To investigate

this hypothesis further, I recommend examining cytokine expression patterns in AR-exposed and mange-infected bobcats in the study area, with special focus on determining whether mange-infested bobcats have a Th2 dominated immune response.

Age class

Animal sex and age influenced multiple hematological parameters, as has been reported in other studies (Brannon 1985a, 1985b, Pérez et al. 2003, Aroch et al. 2007, Yochem et al. 2008), including for other felids (Beltrán et al. 1991), and specifically bobcats (Fuller et al. 1985). Establishing baseline hematological parameters for free-ranging populations can be important for their long-term management (Beltrán et al. 1991). To my knowledge, I am the first to report hematological parameters for urban bobcat populations and bobcat kittens.

Perhaps most interesting and novel, I observed age-associated differences in lymphocyte populations and neutrophil populations. Immune competence is known to decrease with age in multiple taxa (Weng 2006, Heaton et al. 2012), although immunosenescence in free-ranging wildlife populations has only recently begun to be examined, primarily in birds (e.g. (Cichon et al. 2003, Saino et al. 2003, Hayward et al. 2009, Palacios et al. 2011, Ujvari and Madsen 2011). In the present study, adult bobcats had significantly lower percentages of helper T cells and B In the present study, adult bobcats had significantly lower percentages of helper T cells and B cells that co-expressed CD5, and higher neutrophil counts. Similar patterns occur in humans and domestic cats and increases in granulocytes (e.g. neutrophils) are hypothesized to be compensatory for diminished adaptive immunity (Utsuyama et al. 1992, Paganelli et al. 1992, Heaton et al. 2002). In Soay sheep (*Ovis aries*), immune senescence increases susceptibility to helminth infestation, and this relationship is exacerbated by environmental stressors (Hayward et

al. 2009). In urban bobcat populations, environmental stressors such as habitat fragmentation, urban interface, and toxicant exposure could similarly exacerbate immunological aging and remodeling. Susceptibility to mange infection, however, does not appear to be age-associated, as mange has led to substantial mortalities across all age classes (kittens, juveniles, and adults; Riley et al. NPS unpublished data, Serieys et al. unpublished data).

Age class was also an important predictor of serum chemistry parameters, including for parameters reflective of development in young animals. For example, alkaline phosphatase (ALP) was, on average, approximately 3 times greater in juveniles than adults. Calcium and phosphorus levels were also higher in juveniles than adults. Interestingly, ALP, calcium and phosphorus concentrations are associated with age because they are linked with bone development in young animals (Seal et al. 1975, Pérez et al. 2003, Willard and Tvedten 2004, Aroch et al. 2007, Yochem et al. 2008, May-Junior et al. 2009). I also observed lower creatinine values in juveniles compared with adults. Creatinine levels are documented to increase with age and body weight in some carnivores, including domestic cats (Gleich and Hartmann 2009), reportedly in correlation with muscle mass (Brannon 1985b).

Sex

Sex significantly influenced hematological parameters, potentially as a result of differential capture stress experienced by males compared with females. Previous studies have demonstrated that capture stress can influence hematological parameters, especially leukocyte counts and red blood cell values (Brannon 1985a, Kocan et al. 1985, Marco and Lavín 1999, Serieys et al. 2013). Marco et al. (1999) suggested that catecholamine, or epinephrine, release during stressful physical restraint causes the spleen to contract, increasing red blood cell counts,

hemoglobin, and hematocrit. Transient leukocytosis can also be caused by catecholamine release (Jain 1993). I observed leukocytosis and elevated RBC values in males compared with females, suggesting that males may experience greater capture stress than females. Interestingly, Iberian lynx (*Lynx pardina*) males exhibited leukocytosis, which was also attributed to greater capture stress in males (Beltrán et al. 1991).

Differences in RBC values and serum creatinine between males and females has also been attributed to sexual dimorphism, and specifically weight dimorphism, in some species, including Iberian lynx (*Lynx pardina*; (Beltrán et al. 1991). As with Iberian lynx, male bobcats are approximately 25% heavier than female bobcats in my study area (Riley et al. NPS unpubl. data, Serieys unpubl. data). Brannon (1985a) found differences in RBC values between the sexes in grizzly bears (*Ursus arctos*) in one study area, and a correlation between body weight and RBC in another study area. Creatinine may be elevated in males as a result of greater body weight, and specifically, muscle mass compared with females (Brannon 1985a, Yochem et al. 2008).

Conservation and management implications

Urbanization presents wildlife with novel stressors including potential widespread and chronic exposure to toxicants and pathogens. Evaluating the effects of these stressors on the health of free-ranging animals is difficult. Thus, our ability to understand the far-reaching effects of toxicants on wildlife populations may be limited consequently hindering the ability of management agencies to make effective conservation guidelines, such as increasing regulations concerning the availability and use of certain toxicants that pose ecological risk. I explored the immunological response of bobcats to AR exposure, and to my knowledge, this is the first

investigation of the immunological consequences of AR exposure for free-ranging populations. My findings point to the importance of taking a comprehensive approach to field studies, pairing field studies with laboratory assays, to understand the critical, yet cryptic, impacts of anthropogenic disturbance on wildlife populations.

Exposure of nontarget wildlife to ARs is increasingly recognized as a widespread conservation issue (Erickson and Urban 2004, US EPA 2008, California Department of Pesticide Regulation 2013), with global recognition of the threat these toxicants may pose to numerous predatory species worldwide (e.g. Eason et al. 2002, Erickson and Urban 2004, Berny and Gaillet 2008, Elmeros et al. 2011). However, the compounds have been generally considered a threat only to those species for which are vulnerable to direct mortality associated with exposure (e.g. Erickson and Urban 2004, McMillin et al. 2008, Gehrt and Riley 2010, Gabriel et al. 2012). My finding of an association between AR exposure and immune function that could increase bobcat susceptibility to notoedric mange suggests that even for species tolerant to the Vitamin K antagonistic properties of the compounds, these toxicants can unexpected effects. Potentially, for a wide array of taxa, there may be similar cryptic immunological consequences of exposure to anticoagulants that could cascade into population-level impacts. Notoedric mange has had significant population impacts in at least one urban area of SMMNRA (Riley et al. 2007) that caused a genetic bottleneck and consequent decreased genetic diversity in the affected population (Serieys et al. in review, see Chapter 1). For bobcats, the consequence of exposure, may thus extend to genetic consequences for some populations, further threatening the long-term viability of populations in urban areas. Mange is increasingly recognized in bobcats across California (Serieys et al. 2013, Stephenson et al. 2013), and severe mange associated with AR exposure may similarly affect mountain lion populations (Riley et al. 2007). Future investigations that

focus on the effects of these, or other, toxicants in free-ranging populations should look beyond exposure prevalence, coupling field surveys with laboratory assays, to evaluate the sublethal, but potentially far-reaching, effects that toxicants may have on nontarget species.

TABLES AND FIGURES

Table 3.1. Health parameter data was available for animals sampled from 2007-2014. Age class information was unavailable for one individual for which immunophenotype data was available. Because blood chemistries were available for only two kittens, these data were excluded from statistical analyses.

		Age Class			Sex		Season	
	Total	Adult	Juvenile	Kitten	Male	Female	Wet	Dry
Immunophenotypes	63	42	20	0	39	24	27	36
Complete Blood Counts	116	73	35	6	66	48	56	59
Serum Chemistries	114	74	38	2	68	46	54	60

Table 3.2. Results of significant univariate regression analyses for hematological parameters. Only results from statistically significant analyses are shown (α = 0.05). Data for kittens were available only for CBC parameters. MCV: mean cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

CBC parameter	Predictor	Assoc.	*P*	OR	OR 95% CI
Leukocytes (K/µl)	Age class (juvenile)	-	0.029	0.86	0.74 – 0.98
	Sex (male)	+	0.023	1.16	1.02 – 1.32
	M. haemominutum	+	0.014	1.19	1.04 – 1.37
Lymphocytes (K/µl)	Age class (kitten)	+	<0.001	2.48	1.81 – 3.30
	Anticoagulant exposure	+	0.020	1.42	1.07 – 1.93
Lymphocytes (%)	Age class (kitten)	+	<0.001	3.39	2.63 – 4.35
	M. haemominutum	-	0.028	0.73	0.55 – 0.95
Neutrophils (K/µl)	Age class (kitten)	-	0.005	>10,000	>10,000
	M. haemominutum	+	0.023	>10,000	>10,000
Neutrophils (%)	Age class (kitten)	-	<0.001	<0.00001	<0.00001
	M. haemominutum	+	0.009	1374.88	7.04 – >10,000
	Anticoagulant exposure	-	0.031	0.002	0.0001 – 0.05
Monocytes (K/µl)	*M. haemominutum*	+	0.033	>10,000	>10,000
Platelets (K/µl)	Age class (kitten)	+	0.006	>10,000	>10,000
	Age class (juvenile)	+	0.001	>10,000	>10,000
	Bartonella sp. exposure	-	0.003	<0.00001	<0.00001
RBC (M/µl)	Age class (kitten)	-	<0.001	0.01	0.04 – 0.21
	Sex (male)	+	0.029	1.53	1.05 – 2.24
	Wet season	-	0.009	0.59	0.40 – 0.87
Hemoglobin (g/dl)	Age class (kitten)	-	<0.001	0.05	0.02 – 0.01
	Sex (male)	+	0.047	1.72	1.01 – 2.95
	Wet season	-	0.020	0.59	0.31 – 0.90
Hematocrit (%)	Age class (kitten)	-	<0.001	0.76	0.65 – 0.86
	Sex (male)	+	0.023	1.06	1.01 – 1.12
	Wet season	-	0.001	0.91	0.87 – 0.96
MCV (fl)	Age class (kitten)	+	0.031	1.09	1.01 – 1.17
MCH (pg)	Age class (kitten)	+	0.003	1.09	1.03 – 1.16
MCHC (g/dl)	Wet season	+	0.003	4.13	1.63 – 10.46

Table 3.3. Results of significant univariate regression analyses for immunophenotypes. The p-value, association (Assoc.), odds ratio (OR) and 95% confidence interval for each odds ratio are shown. Only results from statistically significant analyses are shown ($\alpha = 0.05$).

Immunophenotype parameter	**Predictor**	***P***	**Assoc.**	**OR**	**OR 95% CI**
Helper T lymphocytes (CD5+CD4+CD8-) (%)	Age class (juvenile)	0.005	+	1.32	1.09 – 1.59
Activated cytotoxic T cells (CD5+CD25+CD8+) (%)	Wet season	0.034	-	0.33	0.10 – 0.70
Total B lymphocytes (CD21+) (cells/µl)	FIV	0.047	+	1.65	1.00 – 2.77
	M.haemominutum	0.011	-	0.47	0.23 – 0.77
	Anticoagulant exposure	0.004	+	2.07	1.33 – 3.62
	Percent natural area	0.011	-	0.43	0.23 – 0.82
Total B lymphocytes (CD21+) (%)	FIV	0.022	+	1.45	1.05 – 1.98
	Anticoagulant exposure	0.029	+	1.35	1.04 – 1.78
	Percent natural area	0.001	-	0.52	0.36 – 0.77
CD5+ B lymphocytes (cells/µl)	FIV	0.018	+	1.86	1.13 – 3.23
CD5+ B lymphocytes (%)	Age class (juvenile)	0.039	+	1.35	1.01 – 1.80
	FIV	0.002	+	1.73	1.25 – 2.42
CD5+CD4+CD8+ lymphocytes (%)	Percent natural area	0.021	+	5.00	1.55 – 33.45

Table 3.4. Results of significant univariate regression analyses for serum chemistry values. Only results from statistically significant analyses are shown ($\alpha = 0.05$).

Chemistry parameter	Predictor	*P*	Assoc.	OR	OR 95% CI
Alkaline phosphatase (U/l)	Age class (juvenile)	<0.001	+	3.34	2.13 – 6.45
	M. haemominutum	0.013	-	0.49	0.24 – 0.79
Alanine transaminase (U/l)	Wet season	0.020	+	1.19	1.03 – 1.38
	B. henselae	0.036	-	0.83	0.69 – 0.99
Aspartate aminotransferase (U/l)	Wet season	0.007	+	1.39	1.10 – 1.78
Albumin (g/dl)	Sex (male)	0.033	-	0.89	0.80 – 0.99
	M. haemominutum	0.031	-	0.88	0.79 – 0.99
Total protein (g/dl)	Age class (juvenile)	0.011	-	0.19	0.67 – 0.95
	Wet season	0.040	-	0.84	0.71 – 0.99
Globulin (g/dl)	Age class (juvenile)	0.021	-	0.92	0.87 – 0.99
	Wet season	0.034	-	0.94	0.88 – 0.99
	M. haemominutum	0.036	+	1.07	1.01 – 1.15
Total bilirubin (mg/dl)	Sex (male)	0.014	+	1.06	1.01 – 1.11
	Anticoagulant exposure	0.032	-	0.80	0.64 – 0.98
	Percent natural area	0.010	+	1.12	1.09 – 1.23
Creatinine (mg/dl)	Age class (juvenile)	<0.001	-	0.63	0.52 – 0.75
	Sex (male)	0.046	+	1.18	1.01 – 1.39
	M. haemominutum	0.004	+	1.29	1.09 – 1.53
	Bartonella sp. exposure	0.026	+	1.22	1.02 – 1.46
	T. gondii exposure	0.015	+	1.32	1.04 – 1.62
BUN/Creatinine ratio	Age class (juvenile)	<0.001	+	1.62	1.35 – 1.96
	M. haemominutum	0.042	-	0.77	0.60 – 0.98
Calcium (mg/dl)	Age class (juvenile)	<0.001	+	1.05	1.03 – 1.07
	Sex (male)	0.033	-	0.97	0.95 – 0.99
	M. haemominutum	<0.001	-	0.64	0.52 – 0.78
	Bartonella sp. exposure	0.001	-	0.66	0.53 – 0.83
Phosphorous (mg/dl)	Age class (juvenile)	<0.001	+	2.98	1.96 – 4.52
	FIV	0.022	+	2.11	1.12 – 3.96
	M. haemominutum	<0.001	-	0.35	0.23 – 0.55
	Bartonella sp. exposure	<0.001	-	0.33	0.20 – 0.54
	Anticoagulant exposure	0.029	+	1.74	1.07 – 2.85
	Percent natural area	<0.001	-	0.19	0.08 – 0.43
Sodium (mEq/l)	*Bartonella* sp. exposure	0.019	-	0.99	0.98 – 1.00

Table 3.5. Mean, standard error (SE), and median for hematological and serum chemistry parameters that differed significantly by age group. Samples from kittens were not available to immunophenotype or evaluate blood chemistries. Only those values that were significant are shown.

	Adult			Juvenile			Kitten			ISIS*	
Parameter	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**
Helper T cells (cells/µl)	40	571.1 (76.8)	549.1	19	831.8 (138.1)	574.4	--	--	--	--	--
Helper T cells (%)	41	33.95 (2.1)	37.1	19	44.92 (3.4)	49.3	--	--	--	--	--
CD5+ B cells (%)	44	0.4 (0.03)	0.4	18	0.6 (001)	0.6	--	--	--	--	--
Leukocytes (K/µl)	73	14.1 (0.6)	13.4	35	12.1 (0.6)	11.3	6	10.5 (0.9)	0.9	135	6.9 (0.4)
Lymphocytes (K/µl)	73	1.8 (0.2)	1.5	35	1.8 (0.2)	1.6	6	4.41 (1.01)	1.01	129	2.1 (0.2)
Lymphocytes (%)	73	13.2 (1.0)	11	35	15.8 (1.5)	14	6	44.9 (10.4)	10.4	--	--
Neutrophils (K/µl)	73	10.9 (0.5)	10.1	35	9.3 (0.7)	8.8	6	5.7 (1.5)	1.5	123	4.2 (0.4)
Neutrophils (%)	73	79.2 (1.5)	83	35	75.5 (2.0)	78	6	51.1 (10.2)	10.2	--	--
Platelets (K/µl)	73	413.2 (16.0)	412	35	487.5 (17.6)	500.5	6	607.2 (60.7)	60.7	73	407.0 (16.7)
RBC (M/µl)	73	7.9 (0.1)	7.9	35	8.1 (0.2)	8.1	6	5.6 (0.3)	0.3	132	15.7 (2.1)
Hematocrit (%)	73	12.7 (0.2)	12.6	35	12.9 (0.2)	12.7	6	9.7 (0.4)	0.4	17	42.1 (1.4)
Hemoglobin (g/dL)	73	39.5 (0.6)	39.1	35	39.8 (0.9)	40.2	6	29.9 (1.6)	1.6	16	13.7 (0.3)
MCV (fl)	73	49.9 (0.5)	50	35	48.9 (0.6)	49	6	54.3 (4.6)	4.6	13	46.7 (1.2)
MHC (pg)	73	16.04 (0.1)	16	35	15.9 (0.2)	15.9	6	17.5 (1.3)	1.3	13	15.1 (0.3)
Alkaline phosphatase (U/l)	74	16.7 (1.9)	11.5	38	55.6 (8.8)	40.5	--	--	--	18	55.0 (12.0)
Total protein (g/dl)	74	7.3 (0.1)	7.25	35	7.02 (0.1)	7	--	--	--	15	7.0 (0.2)
Globulin (g/dl)	65	3.8 (0.1)	3.8	29	3.5 (0.1)	3.5	--	--	--	15	3.2 (0.2)
Creatinine (mg/dl)	74	1.9 (0.1)	1.8	38	1.2 (0.1)	1	--	--	--	14	2.0 (0.2)
BUN/Creatinine ratio	74	21.6 (1.3)	19.2	38	35.04 (2.8)	29.3	--	--	--	--	--
Calcium (mg/dl)	74	9.3 (0.1)	9.3	38	9.8 (0.1)	9.8	--	--	--	18	10.2 (0.1)
Phosphorus (mg/dl)	74	5.6 (0.1)	5.8	38	6.7 (0.2)	6.8	--	--	--	17	5.6 (0.3)

*2011, International Species Information System (ISIS), Apple Valley, Minnesota 55124-8151, USA.

Table 3.6. Results of significant multivariate regression analyses for all health parameters. Only significant model parameters, determined using backward stepwise selection, are shown ($\alpha = 0.05$).

Health parameter	Additive model	Predictor	*P*	Assoc.	OR	OR 95% CI
B lymphocytes (cells/ul)	FIV + Anticoagulant exposure	FIV	0.039	+	1.64	1.02 – 2.63
		Anticoagulant exposure	0.027	+	1.74	1.10 – 2.98
Leukocytes (K/µl)	Sex + *M.haemominutum*	Sex (male)	0.011	+	1.22	1.06 – 1.42
		M.haemominutum	0.009	+	1.20	1.05 – 1.37
Lymphocytes (K/µl)	Age class + Anticoagulant exposure	Age class (kitten)	<0.001	+	3.40	1.67 – 5.62
		Anticoagulant exposure	0.008	+	1.51	1.12 – 2.07
Neutrophils (%)	*M.haemominutum* + Anticoagulant exposure	*M.haemominutum*	0.025	+	471.34	2.40 – 9273.37
		Anticoagulant exposure	0.011	-	0.009	0.000004 – 0.18
Alanine transaminase (U/L)	Season + *B.henselae* infection	Wet season	0.007	+	1.27	1.07 – 1.50
		B.henselae	0.025	-	0.82	0.70 – 0.98
Albumin (g/dL)	Sex + *M.haemominutum*	Sex (male)	0.049	-	0.89	0.79 – 0.99
		M.haemominutum	0.025	-	0.88	0.79 – 0.98
Total bilirubin (mg/dL)	Sex + Anticoagulant exposure	Sex (male)	0.024	+	1.05	1.01 – 1.10
		Percent natural area	0.011	+	1.12	1.03 – 1.22
Creatinine (mg/dL)	Age class + *T.gondii*	Age class (juvenile)	<0.001	-	0.64	0.51 – 0.78
		T.gondii	0.021	+	1.26	1.02 – 1.52
Calcium (mg/dL)	Age class + *Bartonella sp.* exposure + *M.haemominutum*	Age class (juvenile)	<0.001	+	1.04	1.02 – 1.07
		M.haemominutum	0.002	-	0.97	0.95 – 0.99
		Bartonella sp. exposure	0.009	-	0.97	0.95 – 0.99

Figure 3.1. a) Map of Santa Monica Mountains National Recreation Area sampling areas. Circles and triangles represent capture locations for individuals for which physiological data was available. Closed and open circles represent individuals for which anticoagulant exposure data was also available while triangles represent individuals for which anticoagulant exposure data was unavailable. b) Mean absolute counts and percent of B cells partitioned by sampling locations. Bars represent standard errors. Mean B cell counts are highest in areas where anticoagulant exposure detection frequency is highest. MUGU: Point Mugu State Park, MOOR: Moorpark, THOU: Thousand Oaks, MCSP: Malibu Creek State Park, TOPA: Topanga State Park, HLWD: all sampling areas east of the I-405, primarily comprised of the Hollywood Hills.

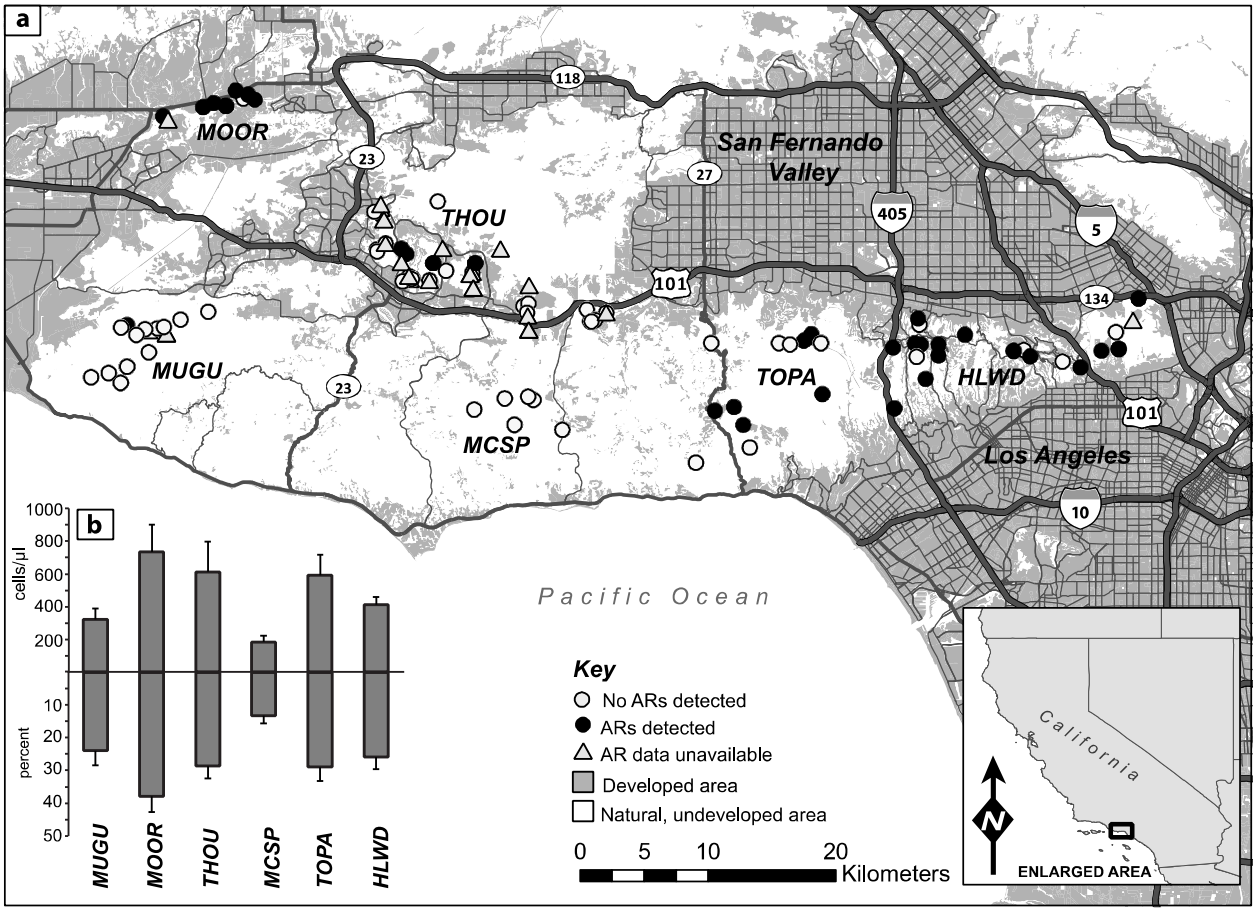


Figure 3.2. Proportion detection of pathogens sampled. Physiological data were available for samples tested during 2007–2011. FCV: Feline calicivirus, FHV: Feline herpes virus, FIV: Feline immunodeficiency virus; FPV: Feline panleukopenia virus.

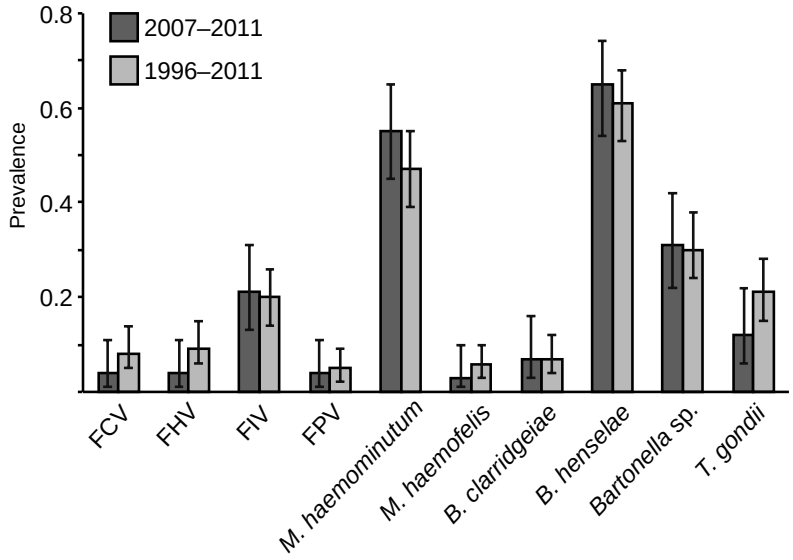
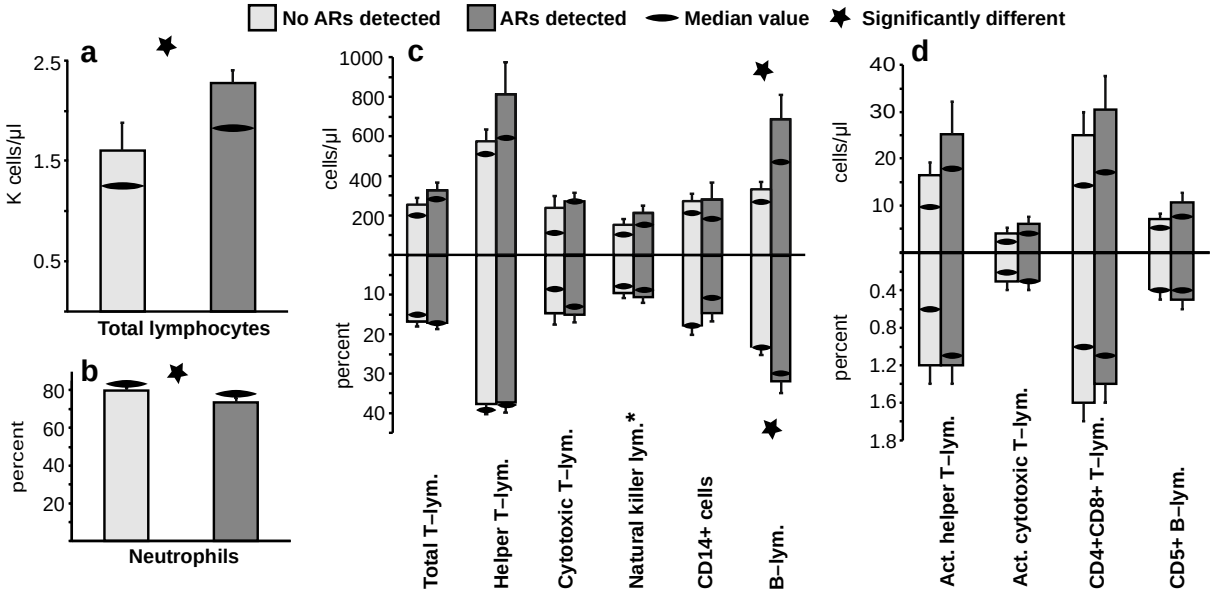


Figure 3.3. Immunological data for bobcats partitioned by detection of anticoagulants in blood samples tested. a) Absolute lymphocyte counts were significantly elevated in bobcats with detectable anticoagulants. b) The percent of neutrophils was significantly lower in bobcats exposed to anticoagulants. c-d) T and B lymphocyte profiles generated using flow cytometry. CD14+ cells represent moncoytes or macrophages that were observed in the live lymphocyte gate. Only absolute counts and the percent of B lymphocytes differed significantly in the two groups. lym.: Lymphocyte, Act.: Activated. *The cytotoxicity of natural killer cells has not been validated with functional assays.



APPENDIX

Table C.1. Disease prevalence estimates for samples collected from 1996-2011. Health parameter data was available for animals sampled from 2007-2011.

			2007-2011		1996-2011	
Disease	**Status**	**Pathogen type**	**N**	**Prop (95% CI)**	**N**	**Prop (95% CI)**
Feline calicivirus (FCV)	Exposure	Virus	93	0.04 (0.01 – 0.11)	171	0.08 (0.05 – 0.14)
Feline herpes virus - 1 (FHV)	Exposure	Virus	93	0.04 (0.01 – 0.11)	171	0.09 (0.06 – 0.15)
Feline immunodeficiency virus (FIV)	Exposure	Virus	87	0.21 (0.13 – 0.31)	179	0.20 (0.14 – 0.26)
Feline panleukopenia (PLV)	Exposure	Virus	93	0.04 (0.01 – 0.11)	171	0.05 (0.02 – 0.09)
Mycoplasma haemominutum	Infection	Bacteria	94	0.55 (0.45 – 0.65)	177	0.47 (0.39 – 0.55)
Mycoplasma haemofelis/turicensis	Infection	Bacteria	94	0.03 (0.01 – 0.10)	177	0.06 (0.03 – 0.10)
Bartonella clarridgeiae	Infection	Bacteria	83	0.07 (0.03 – 0.16)	176	0.07 (0.04 – 0.12)
Bartonella henselae	Infection	Bacteria	93	0.65 (0.54 – 0.74)	176	0.61 (0.53 – 0.68)
Bartonella sp. serology	Exposure	Bacteria	93	0.31 (0.22 – 0.42)	171	0.30 (0.24 – 0.38)
Toxoplasma gondii IgG	Exposure	Protozoa	92	0.12 (0.06 – 0.22)	170	0.21 (0.15 – 0.28)

Table C.2. Mean, standard error (SE), and median immunophenotypes for bobcats with and without detectable anticoagulants in their blood. Parameters in bold were significantly different between the two groups ($\alpha = 0.05$).

	No anticoagulants detected			Anticoagulants detected		
Immunophenotype	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**
Total T cells (cells/µl)	34	255.9 (31.5)	194.5	22	327.4 (38.5)	281.1
Total T cells (CD5+) (%)	35	16.81 (1.2)	15.0	22	17.1 (1.5)	17.4
Helper T cells (cells/µl)	35	574.6 (60.4)	510.1	19	812.8 (160.8)	589.3
Helper T cells (CD5+CD4+CD8-) (%)	36	37.7 (2.61)	39.20	19	37.1 (2.7)	37.9
Cytotoxic T cells (cells/µl)	35	235.9 (60.4)	107.8	19	275.6 (40.6)	276.9
Cytotoxic T cells (CD5+CD8+CD4-) (%)	36	14.7 (3.0)	8.72	19	14.8 (2.1)	13.0
CD4/CD8 Ratio	36	5.47 (0.7)	5.1	19	4.0 (0.7)	2.9
Activated helper T cells (cells/µl)	32	16.5 (2.8)	9.7	19	25.2 (7.0)	17.8
Activated helper T cells (%)	33	1.2 (0.2)	0.6	19	1.2 (0.2)	1.1
Activated cytotoxic T cells (cells/µl)	32	4.1 (1.2)	2.1	19	6.0 (1.6)	3.8
Activated cytotoxic T cells (%)	33	0.3 (0.1)	0.2	19	0.3 (0.1)	0.3
CD5+CD4+CD8+ cells (cells/µl)	35	25.1 (4.8)	14.3	19	30.5 (7.1)	17.0
CD5+CD4+CD8+ cells (%)	35	1.6 (0.2)	1.0	19	1.4 (0.2)	1.1
Natural killer cells (cells/µl)	35	151.4 (30.7)	100.6	19	210.1 (41.9)	146.1
Natural killer cells (CD5-CD4-CD8+) (%)	36	9.6 (1.2)	7.6	19	10.4 (1.6)	8.9
Macrophages/monocytes (cells/µl)	34	269.9 (38.8)	215.1	22	281.3 (82.0)	178.6
Macrophages/monocytes (CD14+) (%)	35	18.0 (2.1)	18.2	22	14.4 (2.4)	10.8
Total B cells (K/µl)*	34	331.8 (39.2)	277.3	22	686.6 (122.2)	459.5
Total B cells (CD21+) (%)*	35	23.3 (2.1)	23.2	22	31.7 (3.3)	29.9
CD5+ B cells (cells/µl)	34	7.1 (1.2)	5.1	22	10.7 (2.1)	7.6
CD5+ B cells (CD5+CD21+) (%)	35	0.4 (0.1)	0.4	22	0.5 (0.1)	0.4

Table C.3. Mean, standard error (SE), and median for hematological and serum chemistry parameters that differed significantly by feline immunodeficiency virus (FIV) exposure or *M. haemominutum* infection. * indicates values for which FIV exposure was a significant predictor. † indicates values for which *M. haemominutum* was a significant predictor. Only values for which FIV or *M.haemominutum* were significant predictors are shown.

	Feline immunodeficiency virus						*Mycoplasma haemominutum*					
	Unexposed			Exposed			Uninfected			Infected		
Health parameter	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**
Total B cells (CD21+) (%)*	32	20.5 (4.5)	20.6	12	29.7 (1.8)	29.1	28	28.5 (2.7)	26.2	23	20.9 (2.4)	20.5
Total B cells (cells/µl)*†	32	314.4 (48.4)	261.9	12	520.9 (11.6)	447.9	28	595.7 (86.6)	413.0	23	280.4 (35.4)	259.4
CD5+ B cells (%)*	32	0.3 (0.0)	0.3	12	0.6 (0.1)	0.4	28	0.4 (0.0)	0.4	23	0.5 (0.1)	0.4
CD5+ B cells (cells/µl)*	32	5.5 (0.9)	4	12	10.2 (2.4)	7.6	28	5.2 (0.7)	4.0	23	10.2 (1.7)	6.8
Leukocytes (K/µl)†	62	14.1 (0.6)	13.1	17	12.5 (1.1)	10.9	49	12.5 (0.6)	11.7	38	14.9 (0.8)	14.4
Lymphocytes (%)†	58	13.3 (1.1)	11	16	16.8 (2.2)	15	46	16.7 (1.3)	16.5	36	12.3 (1.4)	11.0
Neutrophils (K/ul)†	58	11.0 (0.6)	10.1	16	9.1 (0.8)	8.9	46	9.3 (0.6)	9.3	36	11.6 (0.8)	10.6
Neutrophils (%)†	58	78.6 (1.7)	83	16	76.0 (2.7)	78.5	46	74.0 (2.1)	76.0	36	81.2 (1.5)	83.0
Monocytes (K/ul)†	58	0.4 (0.0)	0.4	16	0.4 (0.0)	0.4	46	0.4 (0.0)	0.4	36	0.5 (0.0)	0.5
Alkaline phosphatase (U/l)†	66	26.0 (3.5)	14	17	31.5 (7.9)	19	51	35.7 (4.4)	22	40	17.6 (3.9)	8.5
Albumin (g/dl)†	66	3.4 (0.0)	3.4	17	3.4 (0.1)	3.4	51	3.4 (0.0)	3.4	40	3.3 (0.0)	3.4
Globulin (g/dl)†	54	3.8 (0.1)	3.8	13	3.9 (0.1)	4	40	3.7 (0.1)	3.7	33	4.0 (0.1)	3.9
Creatinine (mg/dl)†	66	1.7 (0.1)	1.6	17	1.5 (0.2)	1.2	51	1.4 (0.1)	1.3	40	1.9 (0.1)	1.7
Calcium (mg/dl)†	66	9.3 (0.1)	9.3	17	9.5 (0.2)	9.3	51	9.6 (0.1)	9.5	40	9.1 (0.1)	9.1
Phosphorous (mg/dl)*†	66	5.9 (0.1)	6	17	6.6 (0.3)	6.4	51	6.5 (0.2)	6.4	40	5.5 (0.2)	5.5

Table C.4. Mean, standard error (SE), and median for hematological and serum chemistry parameters that differed significantly by sex. Only those values that were significant are shown.

	Male			Female			ISIS*	
Parameter	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**	**Median**	**N**	**Mean (SE)**
Leukocytes (K/µl)	66	14.3 (0.6)	13.1	48	12.3 (0.6)	11.4	135	6.9 (0.4)
RBC (M/µl)	66	8.1 (0.1)	8.1	48	7.7 (0.2)	7.7	132	15.7 (2.1)
Hematocrit (%)	66	40.2 (0.8)	40.3	48	37.9 (0.7)	37.3	17	42.1 (1.4)
Hemoglobin (g/dl)	66	12.9 (0.2)	12.9	48	12.3 (0.2)	12.2	16	13.7 (0.3)
Albumin (g/dl)	66	3.4 (0.03)	3.4	48	3.5 (0.04)	3.5	132	3.4 (0.06)
Total bilirubin (mg/dl)	66	0.3 (0.02)	0.3	48	0.2 (0.01)	0.2	136	0.3 (0.02)
Creatinine (mg/dl)	66	1.7 (0.1)	1.6	48	1.5 (0.1)	1.5	14	2.0 (0.2)
Calcium (mg/dl)	66	9.4 (0.1)	9.3	48	9.6 (0.1)	9.6	18	10.2 (0.1)

*2011, International Species Information System (ISIS), Apple Valley, Minnesota 55124-8151, USA.

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