UC Agriculture & Natural Resources
Proceedings of the Vertebrate Pest Conference

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Permalink
https://escholarship.org/uc/item/10p4x6t2

Journal
Proceedings of the Vertebrate Pest Conference, 28(28)

ISSN
0507-6773

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Publication Date
2018

DOI
10.5070/V42811047

Widespread Anticoagulant Poison Exposure is Linked with Immune Dysregulation and Severe Notoedric Mange in Urban Bobcats

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ABSTRACT: Human activities threaten wildlife with a variety of novel stressors such as exposure to toxicants. Anticoagulant rodenticides (ARs) are toxicants applied worldwide and through bioaccumulation, threaten species that prey on poisoned rodents or their predators. We studied a population of urban bobcats in southern California that declined rapidly from 2002-2005 due to notoedric mange. We first assessed prevalence of AR exposure using blood and liver samples across the population and found widespread exposure (>90%). Death associated with mange was strongly correlated with cumulative first- and second-generation AR exposure. These findings suggested that exposure to both first- and second-generation ARs were an underlying cause of the disease. We next aimed to understand the sublethal immunological and physiological effects of AR exposure in this natural population. We used two approaches: 1) we used a comprehensive suite of health assays (complete blood counts, blood chemistry assessment, and immunological profiling), and 2) we quantified AR-induced differential gene expression in blood for a subset of individuals. We found that sublethal AR exposure, primarily measured as exposure to diphacinone, is associated with hallmark indicators of generalized systemic inflammation that in persistence could promote immune dysfunction. Further, differential gene expression findings supported the results of immunological profiling. Further, a decrease in the expression of genes associated with epithelial maintenance simultaneous to a decrease in gene expression linked with ectoparasitic immune response may explain the link between AR exposure and mange vulnerability. Such indirect effects of sublethal exposure exemplify the challenge of protecting wild populations from common toxicants in human-dominated environments.

KEY WORDS: anticoagulant rodenticides, cytokines, diphacinone, gene expression, immune dysfunction, notoedric mange, urbanization

INTRODUCTION

Pesticide exposure is a leading threat to biodiversity globally (McGill et al. 2015). Toxicants used in and around urban and agricultural areas often indiscriminately kill species (Berny 2007, Elliott et al. 2014, Hindmarch et al. 2018). Even when used in small amounts, some toxicants are prone to bioaccumulation that can lead to widespread exposure of many nontarget species (Geduhn et al. 2015). While unintended, direct mortality and sublethal pesticide exposure can influence population dynamics (Thomas et al. 2011) and potentially escalate to ecosystem impacts if the toxicant exposure is pervasive across numerous species (Hindmarch et al. 2018). Yet the sublethal effects of toxicant exposure, which can include impaired immune competence (Ross 2002, Galloway and

Handy 2003) and increased disease susceptibility (Bradley and Altizer 2007, Presley et al. 2010), are difficult to study in natural populations and thus are poorly understood (Berny 2007).

Globally, anticoagulant rodenticides (ARs) are the principal chemical method used for lethal control of rats and mice (Stone et al. 1999). As vitamin K antagonists, ARs slowly deplete vitamin K clotting proteins, causing internal hemorrhage and death up to ten days after lethal ingestion of the poisons (Cox and Smith 1992). ARs are composed of two classes of compounds; first-generation (FGARs; warfarin, diphacinone, chlorophacinone, and coumachlor) and second-generation ARs (SGARs; brodifacoum, bromadiolone, and difethialone). SGARs have prolonged action and increased potency, and with

hepatic half-lives ranging 6-12+ months (Eason et al. 2002), their actual tissue persistence when a sublethal dose is ingested can be greater than six years (Vandebroucke et al. 2008). Consequently, for predatory species that consume prey species targeted with ARs, both acute and chronic secondary toxicant exposure may occur (Serieys et al. 2015a, Hindmarch et al. 2018).

Sublethal AR exposure has been correlated with epizootics of notoedric mange (*Notoedres cati*), an ectoparasitic disease, in bobcat (*Lynx rufus*) populations in California (Riley et al. 2007, Serieys et al. 2013, 2015a). This disease can cause extensive dermatitis resulting in emaciation, secondary systemic problems such as septicemia and hypoglycemia, and changes in activity including weakness, incoordination, and coma (Serieys et al. 2013). Notoedric mange was the greatest source of mortality for bobcats at Santa Monica Mountains National Recreation Area (SMMNRA), a national park in southern California, from 2002-2005 (Riley et al. 2007). The annual survival rate for radio-collared bobcats fell by >50% and in 2003 51% of radio-collared animals died of mange. The epizootic had profound population-level impacts, significantly reducing the effective population size and causing a genetic bottleneck (Serieys et al. 2015b). While susceptibility to severe mange in California bobcats has been linked to repeated, cumulative first- and second-generation AR exposure (Riley et al. 2007, Serieys et al. 2015a), the mechanism by which AR exposure could increase disease susceptibility was unknown until very recently (Fraser and Mouton et al. 2018, Serieys et al. 2018).

Here, we summarize the approaches we took to robustly investigate the breadth and impacts of ARs in the southern California urban bobcat population affected by mange in an effort to understand the potential causal link between ARs and mange. Specifically, we: 1) investigated how widespread AR exposure was in this population (Serieys et al. 2015a), 2) tested the association between anticoagulant rodenticide exposure and death due to notoedric mange (Serieys et al. 2015a), 3) investigated the influence of ARs on the physiological and immunological health of live-captured bobcats (Serieys et al. 2018), and 4) quantified differential gene expression profiles of apparently healthy free-ranging bobcats both exposed, and unexposed, to ARs in SMMNRA (Fraser and Mouton et al. 2018).

METHODS

Our study area was Santa Monica Mountains National Recreation Area (SMMNRA), the eastern boundary of which is less than 10 km from downtown Los Angeles, CA. The park encompasses both large regions of continuous protected habitat with minimal urban development, including state and national park lands, and highly fragmented areas with intense urban development. Long-term urban bobcat research by the National Park Service allowed us to integrate information collected between 1996-2012 on bobcat demography, disease status, population dynamics, mortality, physiological and immunological parameters, gene expression, and AR exposure (see Serieys et al. 2015a, 2018, Fraser and Mouton et al. 2018 for more detailed methods).

We first investigated how widespread AR exposure was in SMMNRA by live-trapping 195 bobcat individuals to collect blood for AR testing between 1996–2012. During this same time period, we additionally sampled liver tissue (the preferred tissue for AR studies) from 172 individuals that were found dead in the study area. The majority (68%) of the deaths documented were radio-collared bobcats, and thus this sample set represents an unbiased dataset of mortality and rodenticide exposure (ie., rodenticide exposure and source of mortality were independent; Serieys et al. 2015a).

Second, we tested the association between death due to notoedric mange and exposure to ARs with a larger, more complete dataset than available in Riley et al. (2007). We used AR results from liver samples only since we did not have any blood samples from bobcats that died with severe mange. Liver samples were also collected between 1996-2012. Seventy individuals died with severe mange and 85 died without mange of other causes (primarily vehicle collisions).

Third, we used a variety of measures to assess the sublethal physiological impacts of AR exposure on urban bobcats (see Serieys et al. 2018 for more details). Briefly, we live-captured 124 bobcats in cages between 2007-2012 to collect antemortem blood samples required for physiological and immunological assessments. From these individuals, we collected blood and serum for use in a variety of assays. Using blood samples (n = 99), we tested for the presence of AR residues for warfarin, coumachlor, bromadiolone, brodifacoum, diphacinone, chloro-phacinone and difethialone, the standard commercial panel available at the time of testing in 2012. We assessed clotting times using two measures: prothrombin times (PT; n = 24) and the more sensitive proteins induced in vitamin K absence (PIVKA; n = 50). To evaluate organ function, we quantified serum chemistry values (n = 116). To measure immune function, we performed complete blood counts (CBC; n = 118) and we adapted immune-phenotyping assays designed for domestic cats to characterize T and B lymphocyte profiles using flow cytometry (immunophenotypes, n = 64). We also measured concentrations of a battery of circulating blood cytokines in 92 bobcats (Serieys et al. 2018).

Fourth, in 52 live-captured individuals also represented in Serieys et al. (2018), we quantified sublethal AR-related effects on genome-wide expression patterns in blood (Fraser and Mouton et al. 2018). Twenty-six individuals were documented exposed to ARs, while 26 individuals were documented as unexposed. This approach allowed us to quantify differential gene expression patterns in exposed versus unexposed bobcats, and to pinpoint specific gene pathways that are up- or down-regulated in response to AR exposure.

To isolate the effects of ARs on immune and gene expression parameters while controlling for disease status and demographic factors, we used a variety of statistical approaches (Fraser and Mouton et al. 2018, Serieys et al. 2018). In brief, because demography (age class and sex), season of sample collection, and disease exposure or infection status may also have strong effects on immune function, we used principal components analysis (PCA) to determine which of these parameters explained significant

variance in our datasets. Next, in Serieys et al. (2018), we used linear models to test for a relationship between each of the immune and physiological parameters and AR exposure, while controlling for age class, *M. haemominutum* infection and *Bartonella sp.* infection (found to influence immune parameters in the PCA). We next tested for predictable, systemic health effects of AR exposure on individuals using a random forest classifier (Serieys et al. 2018). For gene expression analyses (see Fraser and Mouton et al. 2018 for specific details), after quality control filtering, we retained 12,332 genes with sufficient coverage to include in analyses. We next performed gene-by-gene linear mixed models to identify differentially expressed genes in AR-positive bobcats. Next, we assigned all 12,332 genes to functional categories based on the identity of the specific cell types giving rise to observed AR-related differences in whole blood gene expression. Overall, our uniquely comprehensive approach provides a new precedent for integrating pathogen status, life history data and toxicant exposure to predict and understand the immunological health of a free-ranging carnivore, providing new understanding of the cryptic threat posed by chronic AR exposure at the ever-growing urban wildlife interface.

RESULTS AND DISCUSSION
Anticoagulant Rodenticide Exposure is Widespread

We detected widespread AR exposure (to a variety of AR compounds) using both blood and liver tissue. Eighty-eight percent of bobcats had livers with detectable AR residues and 34% of live-captured bobcats had detectable AR residues in their blood (Serieys et al. 2015a). In the most comprehensively sampled individuals (both blood and liver tested, N = 64), 92% of bobcats were exposed to ARs, most frequently to ≥3 compounds indicating multiple exposure events. Although many species such as coyotes die from anticoagulant toxicity through uncontrolled bleeding (Riley et al. 2003), only one or two bobcats in SMMRNA died from anticoagulant toxicosis, suggesting the potential for widespread sublethal, chronic exposure to ARs (Serieys et al. 2015a). Further, two fetal bobcats were already exposed to multiple ARs (2 and 5 compounds). In both cases, both first- and second-generation compounds were detected. AR exposure in bobcats is thus likely a chronic condition that can begin during prenatal development. Further, because bobcat individuals are exposed to multiple types of first- and second-generation compounds, this chronic condition may be a mixture of cumulative and interactive effects of first- and second-generation rodenticides (see next paragraph; Serieys et al. 2015a)

Diphacinone exposure was greater than anticipated based on previous surveys conducted using liver tissue only. Specifically, diphacinone was the primary compound detected in blood (76% of blood AR detection cases). Anticoagulants have considerably shorter blood half-lives than liver half-lives on the order of days *versus* years (Vandebroucke et al. 2008). First-generation diphacinone was detected more than three times as frequently in blood as second-generation compounds brodifacoum or bromadiolone. Liver assays thus underestimate widespread exposure to diphacinone

because of their vastly shorter hepatic half-lives in comparison to SGARs. Yet we still detected widespread diphacinone exposure in liver (40%). Our findings thus indicate that diphacinone is the most pervasive compound in the environment to which bobcats are being exposed. Further, exposure was most frequently to three or more compounds (predominantly exposure to brodifacoum, bromadiolone, and diphacinone). Again, because individuals are exposed to multiple types of first- and second-generation compounds, there may be a mixture of both cumulative and interactive effects of first- and second-generation rodenticides on individual bobcats that can begin during prenatal development and persist for the duration of an animal's lifetime (Serieys et al. 2015a)

Notoedric Mange is Associated with Cumulative First- and Second-generation AR Exposure

In the Serieys et al. (2015a) study, we re-evaluated the relationship between death due to notoedric mange and exposure to ARs after Riley et al. (2007) first described the potential relationship. We tested this association in multiple ways with a substantially larger sample set (155 *versus* 39 bobcats). We investigated the relationship between the number of compounds individuals are exposed to and death due to mange in order to assess potential interactive effects between anticoagulant compounds and increased vulnerability to mange. We also tested the relationship between total residue concentrations and death due to mange. Specifically, the median total residues for bobcats with mange was more than twice the amount (0.52 ppm) as documented in bobcats without mange (0.24 ppm). This difference was significant (p = 0.005; Serieys et al. 2015a). These findings explicitly point to a cumulative impact of all FGAR and SGAR residues in increasing bobcat vulnerability to death due to notoedric mange. We also found strong evidence that exposure to multiple different compounds significantly increased bobcat vulnerability to death due to notoedric mange (Odds ratio: 7.27, $p < 0.001$; Serieys et al. 2015a). For example, 64% of bobcats *without* mange were positive for ≥2 compounds, while 93% of severely mangy bobcats were positive for ≥2 compounds. These findings again point to potential cumulative effects of first- and second-generation AR exposure, but also to potential interactive effects of different compounds in exacerbating bobcat vulnerability to notoedric mange. For example, diphacinone, although classified as a FGAR is an inandione compound unlike warfarin and coumachlor and all SGARs, which are coumarin derivatives. Inandiones are notorious for causing inflammatory diseases, particularly of the skin, and renal dysfunction (Naisbitt et al. 2005). If animals are being routinely exposed to both diphacinone and SGARs, then diphacinone combined with SGARs is likely to have both additive and interactive effects and present a substantial risk to wildlife both directly and indirectly.

To test the potential cumulative and interactive effects of combined FGAR and SGAR exposure, we additionally calculated additional odd ratios using Fisher's exact tests (Table 1). The odds of developing mange when exposed to any first and second-generation compound increases by 25% to 4.39, while when we measure the odds of exposure

Table 1. Results of Fisher's exact tests used to assess the potential relationships between cumulative AR exposure and death due to notoedric mange (versus other sources of mortality). Values in bold show that the odds of death due to mange increase when all residues are considered cumulatively.

Residues detected ≥ 0.05ppm	Comparison	Odds Ratio	95% CI	*P*
Total FGARs & SGARs residues	mange vs. no mange	**4.39**	**1.85-11.41**	**0.0003**
SGARs & diphacinone	mange vs. no mange	**4.00**	**1.67-10.47**	**0.0008**
Only SGARs	mange vs. no mange	3.51	1.56 - 8.47	0.001

only to SGARs, the odds ratio is 3.5 that a bobcat will die with severe mange. We see an increase in the odds of dying from mange when only diphacinone and SGARs are cumulatively assessed.

Anticoagulant Rodenticides Promote Immune Dysfunction

Our most recent studies (Serieys et al. 2018; Fraser and Mouton et al. 2018) describe alarming immune effects of sublethal AR exposure at four levels in free-ranging bobcats: 1) cellular, 2) cellularly derived cell-signaling proteins, 3) biochemical markers of organ function, and 4) gene expression that modulates cascading immunological and physiological processes (see next selection below). These pivotal studies were conducted using a variety of immune and gene expression assays on blood samples collected from bobcats that were all apparently healthy. We specifically excluded samples from bobcats with mange so that mange disease would not add immunological "noise" to our statistical analyses. While we implicate SGARs in these multiple facets of immunological dissonance that we document, because we were limited to AR tests using only blood (not liver), we documented the strong link predominantly between diphacinone exposure and immune consequences for bobcats. Specifically, for every AR detection case (n = 38 cases of AR detection), diphacinone was detected. In only six of 38 AR detections, an additional second-generation compound was also detected. These AR exposure data are unbiased since samples were collected only from free-ranging, live-captured bobcats and all individuals were apparently healthy at the time of capture.

The immune anomalies associated with AR exposure were pervasive and predictable. These AR-induced impacts included cellular changes (e.g., neutrophil, lymphocytes, etc.), changes in cell-signaling protein (ie., cytokines) concentrations that modulate immune cellular function, and changes to biochemical markers of organ function (total bilirubin and phosphorus). Additionally, we found significant overlap in the changes in immune markers that we measured, and changes documented in laboratory studies of rats and humans that are dosed with therapeutic levels of first-generation anticoagulant warfarin. Overall, we found simultaneous evidence of both inflammatory response and immune suppression associated with rat poison exposure that were consistent with laboratory studies and could influence susceptibility to opportunistic infections (see Serieys et al. 2018 for details).

Genome-wide Expression Changes are Associated with AR Exposure

This study provides an unprecedented understanding of the sublethal effects of AR exposure on a wild carnivore, largely because the technology used has become available and feasible for use in ecological studies only very recently. Fifty-two bobcat blood RNA samples were specially preserved at the time of capture and sampling (see Fraser and Mouton et al. 2018 for specific details). The individuals sampled in this study were also represented in the Serieys et al. 2018 study, and thus the amount of life history, physiological, and disease status information available for each individual was in itself unprecedented. This mass of information allowed Fraser and Mouton et al. (2018) to effectively isolate the differential gene expression effects of AR exposure on free-ranging bobcats. As above in Serieys et al. (2018), in all AR detection cases, diphacinone was the primary compound detected. Thus, what associations were made between AR exposure and physiological effects were statistically linked primarily with diphacinone, a first-generation AR. Overall, the tools used to assess physiological states measured by differential gene expression were more sensitive and informative than in Serieys et al. (2018), a study that was limited to more conventional tools for immunological assessments. Overall, Fraser and Mouton et al. (2018) found numerous results concordant with Serieys et al. (2018). However, this study also reported a number of novel findings that help elucidate the potential causal link between ARs and vulnerability to notoedric mange in bobcats.

Fraser and Mouton et al. (2018) identified differential expression of genes involved in xenobiotic metabolism, endoplasmic reticulum stress response, epithelial integrity and both adaptive and innate immune function. Further, we found that differential expression of immune-related genes may be attributable to AR-related effects on leucocyte differentiation. With respect to notoedric mange vulnerability, we were able to put forward several hypotheses based on our myriad results of differential gene expression linked with AR exposure. Specifically, we document simultaneous immune dysregulation and

disruption of epithelial integrity that could predispose bobcats to opportunistic infection by ectoparasitic mange. We found that AR-positive bobcats have substantially reduced expression of genes involved in allergic immune response.

Reduction of these cell types (monocytes and late-stage B lymphocytes) in AR-exposed bobcats suggests that the basic immune machinery needed to protect against severe mange infestation, is compromised by ARs. A simultaneous decrease in expression of cytokines and genes known to be critical in epithelial formation and maintenance furthers suggest that ARs directly affect skin integrity and immunity. We hypothesize that the cumulative effects of these cellular responses to AR exposure increases the susceptibility of individuals to opportunistic parasitism of the skin and inhibits wound healing, allowing for the mange lesions to expand and leading to death.

CONCLUSION

Mounting evidence over more than a decade has shown that anticoagulant rodenticides represent an important conservation concern to nontarget wildlife anywhere these poisons are used. Our long-term, multifaceted research on a population of urban bobcats since 1996 has afforded additional unique understanding of the previously unknown sublethal consequences of chronic first- and second-generation AR exposure on a free-ranging carnivore. Our unprecedented dataset clearly demonstrates that even if not a direct source of mortality, sublethal AR exposure can exert complex systemic physiological and immunological consequences that can cascade into population-level effects. Simultaneous to documenting the widespread and pervasive consequences of AR use on bobcats, other studies in the same region have shown ARs to be a leading source of mortality for sympatric coyotes (Riley et al. 2003, Gehrt et al. 2010), and to also influence mortality in sympatric pumas (Beier et al. 2010).

These cumulative investigations strongly suggest that the indiscriminate nature of these ubiquitous toxicants can not only have population-level effects for some species, but potentially also cascade into ecosystem-wide impacts. Collectively, the unequivocal evidence of multi-dimensional impacts that ARs have on nontarget wildlife populations suggests that despite increasing regulations surrounding the availability and use of ARs to consumers in some U.S. States (such as California), these regulatory steps are inadequate. We have now documented an unanticipated breadth of sublethal consequences associated with cumulative first- and second-generation AR exposure (especially diphacinone exposure) that draw explicit links between ARs, immune and physiological function, and increased vulnerability to an ectoparasitic disease that has cascaded into population level impacts and is spreading to have grave impact across California (Fraser and Mouton et al. 2018, Serieys et al. 2013).

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