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Genome-wide expression reveals multiple systemic effects associated with detection of anticoagulant poisons in bobcats (*Lynx rufus*)

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Abstract

Anticoagulant rodenticides (ARs) are indiscriminate toxicants that threaten nontarget predatory and scavenger species through secondary poisoning. Accumulating evidence suggests that AR exposure may have disruptive sublethal consequences on individuals that can affect fitness. We evaluated AR-related effects on genome-wide expression patterns in a population of bobcats in southern California. We identify differential expression of genes involved in xenobiotic metabolism, endoplasmic reticulum stress response, epithelial integrity and both adaptive and innate immune function. Further, we find that differential expression of immune-related genes may be attributable to AR-related effects on leucocyte differentiation. Collectively, our results provide an unprecedented understanding of the sublethal effects of AR exposure on a wild carnivore. These findings highlight potential detrimental effects of ARs on a wide variety of species worldwide that may consume poisoned rodents and indicate the need to investigate gene expression effects of other toxicants added to natural environments by humans.

KEYWORDS
anticoagulant rodenticides, bobcats, gene expression, secondary poisoning

1 | INTRODUCTION

Poisons aimed at controlling specific pest species may threaten populations of nontarget species. For toxicants that bioaccumulate in the food chain, these threats are greatest to predatory and scavenging species. Although some mortality in nontarget animals occurs via the same molecular pathways that the toxicants are designed to disrupt, sublethal exposure can also have cryptic physiological effects that nonetheless impact individual fitness (Baldwin, Spromberg, Collier, & Scholz, 2009; Gill & Raine, 2014; Santadino, Coviella, & Momo, 2014) and hence may decrease population viability (Rattner, Lazarus, Elliott, Shore, & van den Brink, 2014; Serieys, Armenta et al., 2015; Thompson et al., 2014).

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Anticoagulant rodenticides (ARs) are toxicants used globally to eliminate rodent pests and have been implicated as an important source of mortality in many nontarget species that consume poisoned rodents (Dennis & Gartrell, 2015; Eason, Murphy, Wright, & Spurr, 2001; Fournier-Chambrillon et al., 2004; Gabriel et al., 2015; Huang et al., 2016; Rattner et al., 2014; Sánchez-Barbudo, Camarero, & Mateo, 2012). For example, 81% of tested stone martens (*Martes foina*) and 77% of polecat (*Mustela putorius*) were exposed in Belgium, and between 84% and 100% of birds and other animals tested were exposed in Denmark (Baert et al., 2015; Christensen, Lassen, & Elmeros, 2012; Elmeros, Christensen, & Lassen, 2011). In California, exposure to ARs is a statewide problem with over 70% (368/492) of birds and mammals testing positive for ARs between 1995 and 2011 (California Department of Pesticide Regulation 2013). AR toxicity was a leading cause of mortality in predatory and scavenging birds (Kelly et al., 2014) and in coyotes (*Canis latrans*) (Riley et al., 2003), and it is increasingly recognized as a major threat to the Pacific fisher (*Pekania pennanti*) (Gabriel et al., 2012; Thompson et al., 2014) and to the endangered San Joaquin kit fox (*Vulpes macrotis mutica*) (Nogeire, Lawler, Schumaker, Cypher, & Phillips, 2015). In southern California, over 90% of bobcats and mountain lions (*Puma concolor*) tested positive for ARs (Riley et al., 2007). Further, AR exposure occurs in a wide variety of environments, from pristine areas such as the Sierra Nevada Mountains, to agricultural areas with low human densities such as cattle and horse ranches and grain storage facilities, to urban areas with both high and low-density housing, as well as highly modified areas such as golf courses and natural areas which abut human habitation (Gabriel et al., 2012, 2015; Nogeire et al., 2015; Serieys, Lea, Pollinger, Riley, & Wayne, 2015; Serieys, Armenta et al., 2015).

Several formulations of ARs are currently being used and are grouped into first- and second generation ARs (FGARs and SGARs, respectively). The latter are more acutely toxic, requiring only a single feeding in rodents, and are more persistent in tissue as they were developed as a countermeasure to heritable resistance in rodent populations to FGARs. Both categories of AR's have the same molecular target, VKOR (the enzyme that converts vitamin K to the biologically active form), but SGARs typically have a higher affinity for the enzyme, are more resistant to biotransformation and have a greater bioaccumulation potential (Rattner et al., 2014). The most commonly deployed FGARs are warfarin, chlorophacinone and diphacinone, and the most commonly used SGARs are brodifacoum, bromadiolone, difenacoum and difethialone (US EPA-https://www.epa.gov/rodenticides/restrictions-rodenticide-products). In the Santa Monica Mountains near Los Angeles, CA (USA), bromadiolone and brodifacoum (SGARs) had the highest prevalence of detection in bobcats, whereas diphacinone (FGAR) was detected at the highest concentrations in animal tissues (Serieys, Armenta et al., 2015).

ARs are vitamin K antagonists that reduce vitamin K availability for a variety of critical processes including haemostasis, bone metabolism, angiogenesis, apoptosis, oxidative protein folding and immune function (Danziger, 2008; El Asmar, Naoum, & Arbid, 2014; Esmon, 2005; Ferland, 2012; Li et al., 2003; Opal & Esmon, 2002; Rutkevich & Williams, 2012; Shearer & Newman, 2008; Suttie, 2009). While secondary exposure to ARs frequently leads directly to death from haemorrhaging (California Department of Pesticide Regulation 2013), persistent sublethal exposure appears to be common in nontarget species (Fournier-Chambrillon et al., 2004; Gabriel et al., 2015; Nogeire et al., 2015; Riley et al., 2007). Known side effects of sublethal exposure to vitamin K antagonists in humans and rats include pathologies such as arterial calcification (Danziger 2008), severe skin irritation (Ozcan et al., 2012; Pourdeyhimi & Bullard, 2014) and both immune activation and suppression (Kater, Peppelenbosch, Brandjes, & Lumbantobing, 2002; Popov et al., 2013). Given these potential effects, it is likely that sublethal AR exposure in natural populations disrupts important biological pathways necessary for survival from injury and pathogens.

Here, we analyse global gene expression patterns to evaluate the systemic effects of sublethal AR exposure in wild bobcats living near Los Angeles, California, USA. Bobcats are a highly mobile, widely distributed North American felid and are obligate carnivores that utilize a variety of habitats across their range and have been found even in some urban landscapes (Riley, Boydston, Crooks, & Lyren, 2010). They are highly territorial and solitary, with average home range sizes in our study area of approximately 2.5 km^2 for females and 5.0 km for males (Riley et al., 2010). In the study area, their diets consist primarily of lagomorph and rodent species including cottontail and brush rabits, pocket gophers, ground squirrels and voles, all of which are primary targets of ARs (Bartos et al., 2011; Fedriani, Fuller, Sauvajot, & York, 2000; Riley et al., 2010). Additionally, some nontarget rodents are exposed to ARs, such as woodrats, that are also bobcat prey (Moriarty et al., 2012).

Despite high exposure prevalence in our study area, few bobcat mortalities have been attributed directly to AR toxicity (Riley et al., 2007). However, previous research repeatedly found mortality from notoedric mange (caused by the mite *Notoedris cati*) to be associated with the level of ARs (Riley et al., 2007; Serieys, Armenta et al., 2015), suggesting the potential for sublethal effects of ARs on the ability of bobcats to resist mange mite infection. Mange was the primary source of mortality in the bobcat population from 2002 to 2008 (Riley, Serieys, & Moriarty, 2015; Riley et al., 2010), which resulted in a genetic bottleneck (Serieys, Lea et al., 2015). Notoedric mange had never previously been known to have such severe demographic impacts on any wild felid population, and typically only affected a few individuals that were likely already unhealthy (Pence, Matthews, & Windberg, 1982; Pence, Tewes, Shindle, & Dunn, 1995; Penner & Parke, 1954). The emergence of this epizootic prompted NPS biologists to submit bobcat carcasses to the California Animal Health and Food Safety Laboratory (CAFHS) for necropsy and full evaluation to assess cause of death and any associated factors. Carcass examination and testing for a panel of eight environmental contaminants (lead, manganese, iron, mercury, arsenic, zinc, copper and cadmium) in addition to ARs suggested ARs as the only consistent underlying complication in bobcats that succumbed to death from mange infection (S. P. D. Riley & L. E. K. Serieys,

personal communication, October 31, 2017). However, the mechanism underlying this potential link between mange and AR exposure remains unknown.

By comparing AR-positive cases to those without detectable AR levels, we demonstrate the use of RNA-seq on whole blood to investigate genes and cellular processes that are affected by sublethal AR exposure in bobcats. Based on genes known to interact with vitamin K antagonists (http://ctdbase.org/) (Davis et al., 2017), we expected differential expression of genes involved in haemostasis, xenobiotic metabolism and the immune system. We further sought to identify potential links between altered gene expression and disease susceptibility in bobcats and potentially, other wildlife. To our knowledge, this is the first genome-wide assessment of transcriptional responses to secondary AR exposure in a wild vertebrate population.

2 | MATERIAL AND METHODS

2.1 | Sampling

We conducted our analyses on 52 RNA preserved whole blood samples from bobcats captured as part of an ongoing research project directed by the National Park Service. We selected our samples to include 26 bobcats for which ARs were detected and 26 samples for which ARs were not detected in whole blood at the time of capture (Serieys, Armenta et al., 2015). Additionally, we balanced our samples across sex and age. These bobcats were captured across the Santa Monica Mountains, Simi Hills and Hollywood Hills between 2008 2012 (Figure 1). The study area was comprised of large natural areas within the Santa Monica Mountains, relatively large fragments of natural habitat surrounded by roads and development in the Simi Hills, and intensely urbanized areas in the Hollywood Hills. The dominant natural vegetation types were coastal sage scrub and chaparral. Each animal was captured, processed and sampled in accordance with the Office of Animal Research Oversight of the University of California Los Angeles (Protocol ARC#2007-167-12) and under authorization through California Department of Fish and Wildlife (SC-9791), assessed for AR exposure as described in Serieys, Armenta et al. (2015) and Serieys, Lea et al. (2015) and released at the capture site. Briefly, AR exposure was assessed using high-performance liquid chromatography for the presence and liquid chromatography–mass spectrometry for the quantity of warfarin, coumachlor, bromadiolone, brodifacoum, diphacinone, chlorophacinone and difethialone from tissue, serum or whole blood. Detection of AR exposure in blood can greatly underestimate true exposure prevalence as paired liver

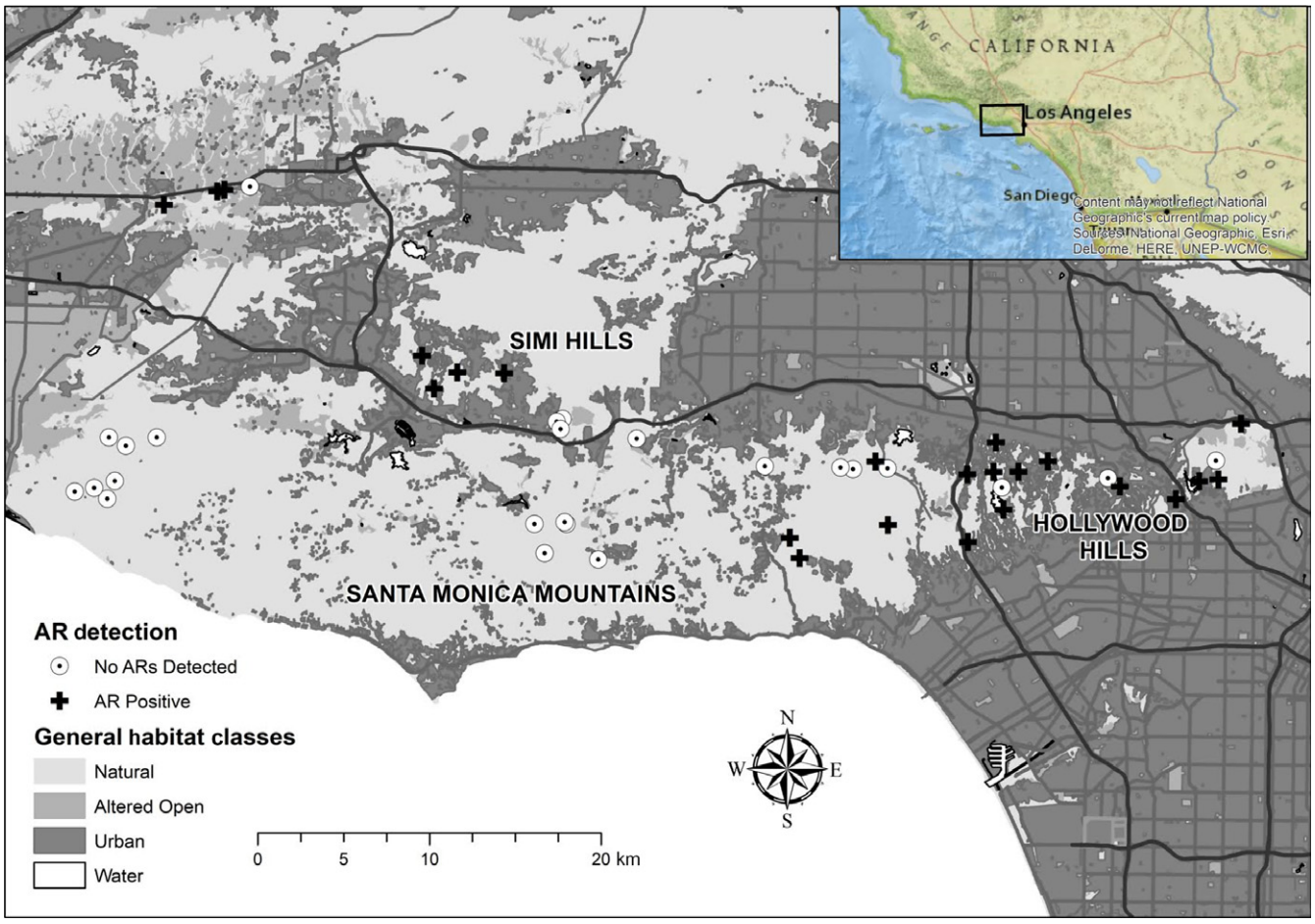

FIGURE 1 Map of the study area depicting sample locations for all 52 bobcats, whether or not the animal tested positive (+) or not positive (⊙) for anticoagulant rodenticides (ARs), and the general land use categories (urban, altered open and natural) [Colour figure can be viewed at wileyonlinelibrary.com]

samples from necropsied animals frequently tested positive for ARs even in the absence of detection in blood (Serieys, Armenta et al., 2015). Several factors may determine the detectability of ARs in blood: time since exposure; the magnitude of exposure; and the metabolic half-life of the AR which is both species and compound specific. Thus, although detection in blood most likely indicates a relatively recent exposure event, we cannot distinguish among all these effector variables. Further, many samples fell below the limit of quantitation but above the level of detection. Hence, we considered AR exposure status as a binary variable (see Serieys, Armenta et al., 2015) and conservatively considered individuals showing detectable levels of at least one and up to five of the seven screened compounds (i.e., >1 ppb) as positive for AR exposure (AR-positive).

All animals in this study were apparently healthy at the time of capture (i.e., no sign of disease). Disease screening was performed at the Center for Companion Animals Studies or in the Feline Retrovirus Research Laboratory in the Microbiology, Immunology and Pathology Department at Colorado State University. Serum samples were analysed separately for Feline Immunodeficiency Virus (FIV) and Puma Lentivirus (PLV) using Western blot. Serum from blood samples was also assayed for Feline Calicivirus (FCV), Feline Herpesvirus (FHV), *Bartonella* sp. and *Toxoplasmosis gondii* specific IgG by enzyme linked immunosorbant assay (ELISA). To test for *Mycoplasma haemofelis*, *M. haemominutum*, *Bartonella henselae* and *Bartonella clarridgeaie* infection, PCR assays were performed on whole blood. Individual animal information is provided in Table S1.

2.2 | Method details

2.2.1 | RNA processing

Total RNA was extracted from 0.5 ml whole blood using the Ambion Mouse RiboPure Blood extraction kit, followed by globin removal using the Ambion GlobinClear Mouse kit (Life Technologies, Inc). RNA was quantified on the Agilent bioanalyzer (Agilent Technologies, USA). RNA integrity number (RIN) scores from globin-depleted RNA samples ranged from 5.5 to 9.3. A minimum of 100 ng was used as input for cDNA library preparation using the Kapa Biosystems stranded mRNA kit (Kapa Biosystems, Ltd). Each sample was uniquely tagged with custom index sequences developed at UCLA (Faircloth & Glen, 2012) comparable to Illumina TruSeq tags. Individual sample libraries were then pooled in equimolar ratios, with 13 or 14 samples per pool and each pool sequenced on two lanes of an Illumina HiSeq 2500 or HiSeq 4000 sequencer (Table S1). Sequencing was performed for 150 bp single-end reads. Library quantification, pooling and sequencing were performed at the Vincent Coates Sequencing Facility at UC Berkeley.

2.2.2 | Quality control, mapping and trimming and read quantification

Raw sequences were processed using Trim Galore! 0.3.1 (Krueger, 2015) to remove Illumina adapters and filter out sequences that did not meet the quality thresholds ($q > 20$, length >25 bp). Alignment of reads was performed on TOPHAT2 2.1.0 (Kim et al., 2013) using the domestic cat (*Felis catus*) as a reference genome (Ensembl release 85.62) (Yates et al., 2016). To maximize the number of unique reads mapped to the reference genome, we used the following parameters: read mismatches 10, max-insertion-length 12, read-edit-dist 22. On average, 70% of reads mapped uniquely, leaving an average of 13,232,179 mapped reads per individual (3,405,189–22,898,827). Summary statistics are available in Table S1.

2.2.3 | Gene expression quantification

Aligned reads were converted to raw counts using HTSEQ (Anders, Pyl, & Huber, 2014) with the "union" mode, resulting in alignment to 21,890 genes. After removal of three globin-related genes (ENSFCAG00000030531, ENSFCAG00000031043, ENSFCAG00000022139) with high expression levels prior to normalization, values for the remaining 21,887 genes were normalized using the trimmed mean of *M*-values (TMM) method in the EDGER package (Robinson & Oshlack, 2010) in R and adjusted for gene length and GC content using custom Python scripts and the package CQN in R (Hansen, Irizarry, & Wu, 2012). The number of genes remaining after filtering for protein-coding genes and sufficient coverage (>10 reads in 75% of cDNA libraries) was 12,332. We used hierarchical clustering of the gene expression adjacency matrix to identify outlier samples (defined as having a Z score >3) with the R package WGCNA (Langfelder & Horvath, 2008).

2.3 | Statistical analysis

A summary of the analyses used in the present paper is available in Figure S1.

2.4 | LIMMA

We performed principal components analysis to identify and remove technical factors from the expression data (Figure S2). Gene-by-gene linear mixed models were used to identify differentially expressed genes in AR-positive bobcats using the LIMMA package in R (Ritchie et al., 2015). We adjusted our significance values to account for multiple hypothesis testing using the false discovery rate (FDR) method as implemented in the QVALUE package in R (Storey, Bass, Dabney, & Robinson, 2015) and selected genes falling below $Q < .05$. We selected the genes falling under a Q-value threshold of .05 and then performed Gene Ontology (GO) analysis on the up and downregulated genes that passed this threshold using g:PROFILER (Reimand et al., 2016). In g:PROFILER (version 1682), we used the 12,332 genes as a statistical background and aligned our significant Ensembl gene ID specifically to the *F. catus* genome. We required a minimum of 2 for the query intersection and applied the Benjamini–Hochberg FDR correction for the significance threshold. The remaining parameters were set using the defaults.

2.5 | WGCNA (weighted gene correlation network analysis)

We assigned all 12,332 genes to functional categories based on coordinated expression patterns using the WGCNA package in R (Langfelder & Horvath, 2008). Briefly, WGCNA searches for genes with similar expression profiles and transforms this correlation matrix into an adjacency matrix via a power function β (Zhang & Horvath, 2005). The adjacency matrix is used to define a measure of node dissimilarity. In conjunction with a clustering method (average hierarchical clustering) and the node dissimilarity measure, the user can identify modules containing highly interconnected genes which can then be related to a trait of interest (Langfelder & Horvath, 2008).

We first ran a *k*-means clustering optimization to determine the most likely number of clusters in our expression data set using the ICGE package in R (Irigoien, Sierra, & Arenas, 2012). In WGCNA, we then followed the automatic, one-step network construction and module detection implemented with the function "blockwiseModules" with an unsigned network algorithm, a power β = 6, corType = bicor, maximum block size = 13000, min module size = 40, mergeCutHeight = 0.5, mergingThresh = 0.5. The remaining parameters were kept at the default setting. This cut-off value yielded the "correct" number of modules, including the "grey" module, which contains genes that are not part of any modules. Subsequently, we performed a hub gene analyses (genes with the highest intramodular connectivity) on each resulting module and submitted the top hub genes (up to 100) for GO analysis using g:PROFILER (Reimand et al., 2016). We used these functional categories based on gene enrichment of biological processes to aid in the interpretation of our linear model results at a systemic level.

In order to assess the stability of the modules and therefore the biological interpretation of the hub gene analyses, we performed a module stability analysis (Langfelder & Horvath, 2012). We conducted 50 full module construction and module detection runs on resampled expression data, where each iteration randomly sampled 52 animals from the original data set, with replacement. Module assignment for each gene was then compared to the original module assignment and overall stability of the hub genes was calculated as the mean proportional assignment of each hub gene to the original module. In addition, we repeated our module detection analysis after changing the correlation type to the default (Pearson) and subsequently calculated module preservation statistics to evaluate whether a given module defined in one data set (reference network) can also be found in another data set (test network) across 200 permutations. Each permutation will report the observed value and the permutation Z score to measure significance, which is then summarized in a composite measure called Z.summary.

2.6 | Transcript origin analysis and transcriptome representation analysis

Transcript origin analysis (TOA) was applied as in Cole, Hawkley, Arevalo, and Cacioppo (2011) to identify the specific cell types giving rise to observed AR-related differences in whole blood gene expression. Transcriptome representation analysis (TRA) was performed as in Powell et al. (2013) to quantify differences in the prevalence of specific cell types based on coordinated shifts in cell type-specific RNA profiles in AR-positive bobcats. Both analyses utilize publicly available leucocyte subset-specific expression profiles as reference distributions to generate cell diagnosticity scores for each gene analysed. The cell diagnosticity scores for AR-associated genes (defined either by fold expression difference [>1.5] or significance [$q < .05$]) are then tested for significant over-representation relative to the basal prevalence of diagnosticity scores across all genes present in the data set (TOA), or the most cell type-diagnostic transcripts are tested for differential expression as a function of AR exposure (TRA). Cell type-specific reference profiles used in the present analyses included major leucocyte subsets (i.e., monocytes, dendritic cells, natural killer cells, B lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, from GEO data set GSE1133), immature/classical (CD16−) vs. mature/nonclassical (CD16+) monocytes (GSE25913), M1 vs. M2 macrophages (GSE51446) and two data sets comparing naïve B lymphocytes with progressively more differentiated B-cell subpopulations (GSE64028 and GSE13411).

3 | RESULTS

3.1 | Principal components of expression data

To evaluate the influence of technical (i.e., batch effects) and biological variables (Table S1) on data structure, we performed linear regression on the principal components (PC) of the normalized read counts. We regressed out technical factors that were significantly correlated with the first PC, including the sequencing platform (HiSeq 2500 or HiSeq 4000), RIN and library preparation. After correcting for technical effects, we found that exposure status was highly significant on PC 1, which explained 19.4% of the total variance (Figure S2). Importantly, none of the pathogens for which each bobcat was currently infected (*Mycoplasma haemominutum*, *M. haemofelis/turricensis*, *Bartonella clarridgeie*, *B. henselae*) were significantly correlated with the first 12 PCs, and although evidence of exposure (seropositivity) to PLV and *Bartonella* spp. was significant on PC 9 (PLV) and PC 12 (*Bartonella*), these principal components explained only 2.6% and 1.9% of the total variation in expression (Figure S2, Table S2). Therefore, differential expression profiles in AR-positive bobcats are not likely due to current infection status for the 10 common feline pathogens (Bevins et al., 2012; Carver et al., 2016) examined. Additionally, age classification (juvenile or adult) was significant on PC 6, which explained only 3.5% of the variance in the data.

3.2 | AR exposure as a linear predictor of differentially expressed genes

To identify genes influenced by AR exposure, we used linear regression to measure fold change (β) and statistical significance (*Q*). Our

data set included read counts for 12,332 genes that were retained after normalization and low coverage filtering. After applying a FDR corrected for multiple testing (Figure S3), a total of 1,783 genes were significantly ($Q < .05$) predicted by exposure status, of which 530 were downregulated and 1,253 were upregulated (Figure 2; Table S3). Eighteen of these genes identified in our model overlap with genes listed in the Comparative Toxicogenomics Database (Davis et al., 2017) as interacting with warfarin, although the direction of dysregulation was not consistent for all genes with responses observed in rats or humans (Table 1).

Downregulated genes were enriched for several GO terms related to immune function, including response to IL-12 and IL-6; positive regulation of acute inflammatory response; complement-mediated cytotoxicity; myeloid differentiation; monocyte activation;

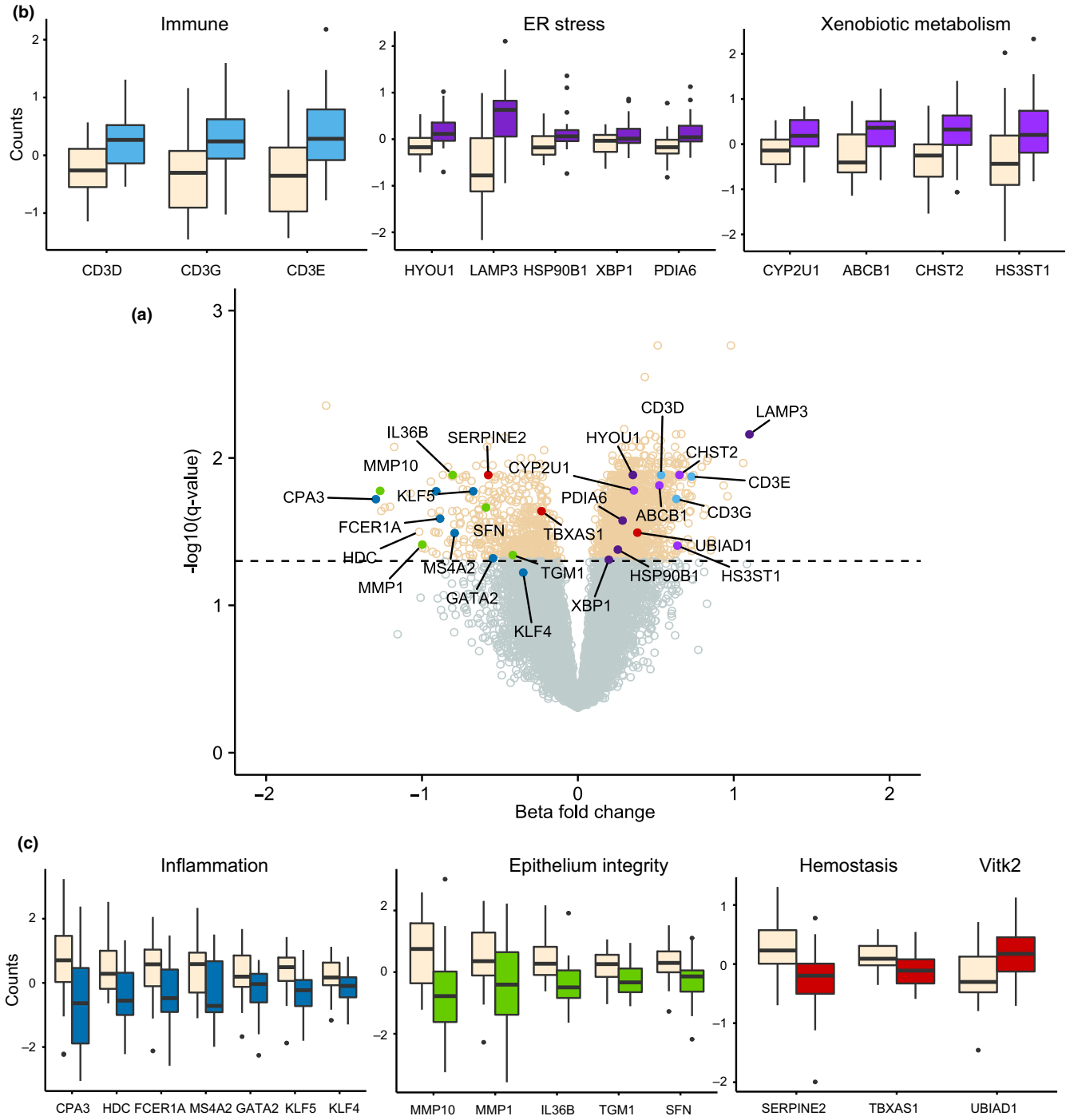

FIGURE 2 (a) Volcano plot depicting the −log10 of the Q value against the β fold change for all 12,332 genes. Significant gene ($Q < .05$) are highlighted in tan. Labelled genes are colour coded by associated physiological process (depicted in b–c). Mean normalized counts of upregulated genes (b) and downregulated genes (c) shown for anticoagulant rodenticide (AR)-negative (light colour) and AR-positive (dark colour) bobcats [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Differential expressed genes listed as related to warfarin in the comparative toxicogenomic database

Gene name	Gene symbol	Known interactions with warfarin	Present study (bobcats)	Beta fold change (β)
ATP-binding cassette subfamily B member 1	*ABCB1*	ABCB1 polymorphism affects the susceptibility to Warfarin ABCB1 protein affects the metabolism of warfarin	↑	.522664633
Adenosylhomocysteinase	*AHCY*	↓	↑	.182681548
BCL2, apoptosis regulator	*BCL2*	↓ (Vitamin K2 inhibit the interaction and increase expression)	↑	.497313399
Chaperonin containing TCP1 subunit 5	*CCT5*	↑	↑	.30416539
Eukaryotic translation initiation factor 3 subunit I	*EIF3I*	↓	↑	.240188572
Ectonucleotide phosphodiesterase 1	*ENPP1*	↑	↑	.835102761
G3BP stress granule assembly factor 1	*G3BP1*	↑	↑	.224464977
Heat shock protein 90 alpha family class B member 1	*HSP90AB1*	↓	↑	.344576673
Heat shock protein family A (Hsp70) member 8	*HSPA8*	↑	↑	.340538007
Keratin 18	*KRT18*	↓	↑	.380455519
NmrA like redox sensor 1	*NMRAL1*	↑	↑	.384219801
Nucleobindin 1	*NUCB1*	↑	↑	.251636364
Proliferation-associated 2G4	*PA2G4*	↑	↑	.321882064
Protein disulphide isomerase family A member 3	*PDIA3*	↑ ↓	↑	.227102201
Ribosomal protein L27	*RPL27*	↑	↑	.314549077
Selenophosphate synthetase 1	*SEPHS1*	↑	↑	.319143095
Tumour protein P53	*TP53*	Affect the expression Increase degradation of TP53 protein	↑	.336253754
U2 small nuclear RNA auxiliary factor 2	*U2AF2*	↑	↑	.187906376

↑ = upregulated, ↓ = downregulated

FC-epsilon receptor signalling; and positive regulation of macrophage chemotaxis. Downregulated genes were also enriched for terms related to epithelium including keratinocyte proliferation, glomerulus development and intestinal epithelial differentiation, and for terms related to vascular processes including Tie-signalling, negative regulation of vasoconstriction, regulation of angiotensin levels in blood, negative regulation of blood circulation and platelet aggregation. Additional terms related to cell cycle, biosynthetic processes, metabolism, reproductive processes and transport (Figure 3a; Table S4).

We observed downregulation of several genes related directly to wound healing and epithelial integrity, including matrix metallopeptidase 1 (*MMP1*: $\beta = -.99$; $Q = .038$) and matrix metallopeptidase 10 (*MMP10*: $\beta = -1.26$; $Q = .01$); as well as two important transcription factor involved in white blood cell production and differentiation, GATA binding protein 2 (*GATA2:* $\beta = -.54$; $Q = .047$) and kruppel-like factor 5 (*KLF5:* $\beta = -.67$; $Q = .016$). Further several genes involved in the allergic response were downregulated. These included membrane spanning 4-domains A2 (*MS4A2:* $\beta = -.79$; $Q = .03$) and Fc Fragment of IgE Receptor Ia (*FCER1A:* $\beta = -.88$; $Q = .025$), encoding for the high affinity IgE beta and alpha receptors, and carboxypeptidase A3 (*CPA3:* $\beta = -1.29$; $Q = .019$) which is involved in granulocytic mediated inflammation. Bobcats exposed to ARs thus may experience a depressed inflammatory response coupled with diminished epithelial integrity and wound healing response.

There were 2.36 times as many upregulated genes, which were enriched for GO terms related predominantly to immune function, specifically to T lymphocytes, as well as terms for gene expression and RNA processing. Immune-related terms included positive regulation of immune response, T-cell differentiation, thymocyte aggregation and T-cell receptor signalling (Figure 3b; Table S5). Notably, we also observed upregulation of UbiA prenyltransferase domain containing 1 *UBIAD1* ($\beta = .38$; $Q = .032$), a mammalian gene involved in the biosynthesis of vitamin K2 (Meehan & Beckwith, 2017; Nakagawa et al., 2010), as well as several genes involved in xenobiotic metabolism including cytochrome P450 family 2 subfamily U member 1 (*CYP2U1*: $\beta = .35$; $Q = .016$), ATP-binding cassette subfamily B member 1 (*ABCB1*: $\beta = .52$; $Q = .015$), carbohydrate sulfotransferase 2 (*CHST2*: $\beta = .65$; $Q = .013$) and heparan sulphate-glucosamine 3-sulfotransferase 1 (*HS3ST1*: $\beta = .64$; $Q = .039$). These results suggest

FIGURE 3 Treemap of the gene ontology (GO) Biological Processes for the down (a) and up (b) regulated genes ($Q < .05$). Box size correlates to the –log10 *p*-value of the GO term enrichment. Boxes with the same colour represent higher level categories of processes. Main Abbreviations: (+): positive regulation, (−): negative regulation, macroph: macrophage. See Table S4 and S5 for GO term details [Colour figure can be viewed at wileyonlinelibrary.com]

that ARs may activate the adaptive immune system as well as processes associated with xenobiotic metabolism and, potentially, responses to vitamin K deficiency. Other GO terms included gene expression, RNA metabolic process, translation, positive regulation of RNA splicing, response to dsRNA and ribonucleoprotein complex biogenesis (Figure 3b; Table S5). Several of the genes in these terms relate specifically to immune and cellular stress responses, likely reflecting increased transcriptional activity due to immune activation and toxicant metabolism.

Further, we observed differential expression of several interleukin cytokines (ILs) in AR-positive bobcats (Table S6). Downregulated IL genes were generally regulators of inflammation including *IL13* ($\beta = -.9$; $Q = .016$) and *IL36B* ($\beta = -.8$; $Q = .013$); whereas upregulated IL genes were generally indicators of B- and T-cell activity, including *ILF2* ($\beta = .24$; $Q = .044$), *ILF3* ($\beta = .25$; $Q = .033$) and *IL7R* ($\beta = .6$; $Q = .017$). Overall, the up- and downregulation of numerous cytokines demonstrate a pronounced dysregulation of critical mediators of immune function, implying both immunosuppressive and stimulating effects of AR exposure.

3.3 | Transcript origin analysis and transcriptome representation analysis

To identify and quantify cellular subsets that contribute to differential gene expression in AR-positive bobcats, we applied a TOA and TRA. The TOA analyses of major leucocyte subsets showed that AR-downregulated genes originated disproportionately from monocytes (CD14+ cells) whereas upregulated genes originated primarily from helper (CD4+CD8−) and cytotoxic (CD4−CD8+) T cells and CD19+ B cells (Table 2). Further, TRA analyses indicated an average 6.4% reduction in total monocyte prevalence within circulating blood of AR-positive bobcats (mean TRA log2 prevalence ratio for monocyte-diagnostic genes = $-0.102 \pm SE$ 0.047, $p = .039$). These results were consistent regardless of whether the differential expression analysis was assessed by effect size (0.917 fold-change) or as a function of the significance threshold ($Q < .05$; 0.952-fold change).

Subsequent TOA analysis focusing on specific monocyte subsets showed that AR-downregulated genes derived predominantly from CD16− (immature "classical") monocytes whereas AR-upregulated genes derived predominantly from CD16+ (mature, "nonclassical") monocytes. Again, these results were consistent regardless of whether differential expression was defined by effect size or statistical significance. In terms of patterns for B cells, TOA analyses of distinct B-cell differentiation states linked AR exposure to a shift towards immature, naive B cells; whereas downregulated genes derived predominantly from more mature/memory B-cell phenotypes, including plasma cells whose primary role is the secretion of antibodies, indicating that these cells were less common or less active or both (Table 2). In general, these results indicate that AR exposure may affect immune function by impacting the relative abundance of circulating immune effector cells and cell-subsets.

TABLE 2 Transcript origin analysis for leucocytes and leucocyte subsets

	p Value	
Cell type	**FD >1.5**	**FD <0.67**
PBMC	*N* = 108	*N* = 149
CD14 monocytes	0.998	0.004*
BDCA4 dendritic cells	0.999	0.999
CD56 NK cells	<0.0001*	0.018*
CD4 T cells	0.002	0.556
CD8 T cells	<0.0001*	0.038*
CD19 B cells	<0.0001*	0.038*
Monocytes	*N* = 76	*N* = 105
CD14+16−	0.992	0.0008*
CD14+16+	0.0072*	0.999
B cells- naïve vs. memory	*N* = 194	*N* = 252
Human_IgM+IgD+CD27+	0.0254*	0.070
Human_class switched	0.999	0.655
Human_IgM+IgD−CD27+	0.058	<0.0001*
Human_IgM+IgD+CD27−	0.006	0.998
B cells- class switched	*N* = 117	*N* = 151
Naïve	0.427	0.738
IgM	0.339	0.819
Switched mem. B cells	0.964	1
Plasma cells	0.889	0.0006*

FD, fold change; PBMC, peripheral blood mononuclear cell; *N*, number of genes.
*Significant.

3.4 | Weighted gene coexpression network analysis

We implemented a WGCNA to assign all 12,332 genes to modules based on patterns of coordinated expression, resulting in 11 modules, including a nonspecific module (Table S3) which was consistent with the *k*-means clustering results (Figure S4). We subsequently assigned each module to functional categories based on GO enrichment analysis of modular hub genes and assessed how many significantly differentially expressed genes (based on the linear model) were assigned to each module (Figure 4a). The dominant expression profile (eigengene) for two of the 10 modules was significantly correlated ($p < .05$) with exposure after FDR correction (Figure 4b,c). Functionally, these modules related to T-cell activation and signalling (Pearson's $r = .46$, $p_{adjusted} = .006$; light blue module) and the inflammatory response (Pearson's $r = -.39$, $p_{adjusted} = .025$; blue module). In addition, four of the remaining eight modules had an overlap of 10 or more genes that were significant in the linear model. These modules were enriched functionally for transferase activity (green module), wound healing/coagulation (red module), endoplasmic reticulum stress response (purple module) and heme metabolic process (yellow module). Module stability for these six modules ranged from 27% to 98%. The hub genes were reassigned to the original module at 98% for the light blue module, at 88% for the green module, at 96% for the red module, at 78% for the yellow module, at 57% for the blue module, at 27% for the

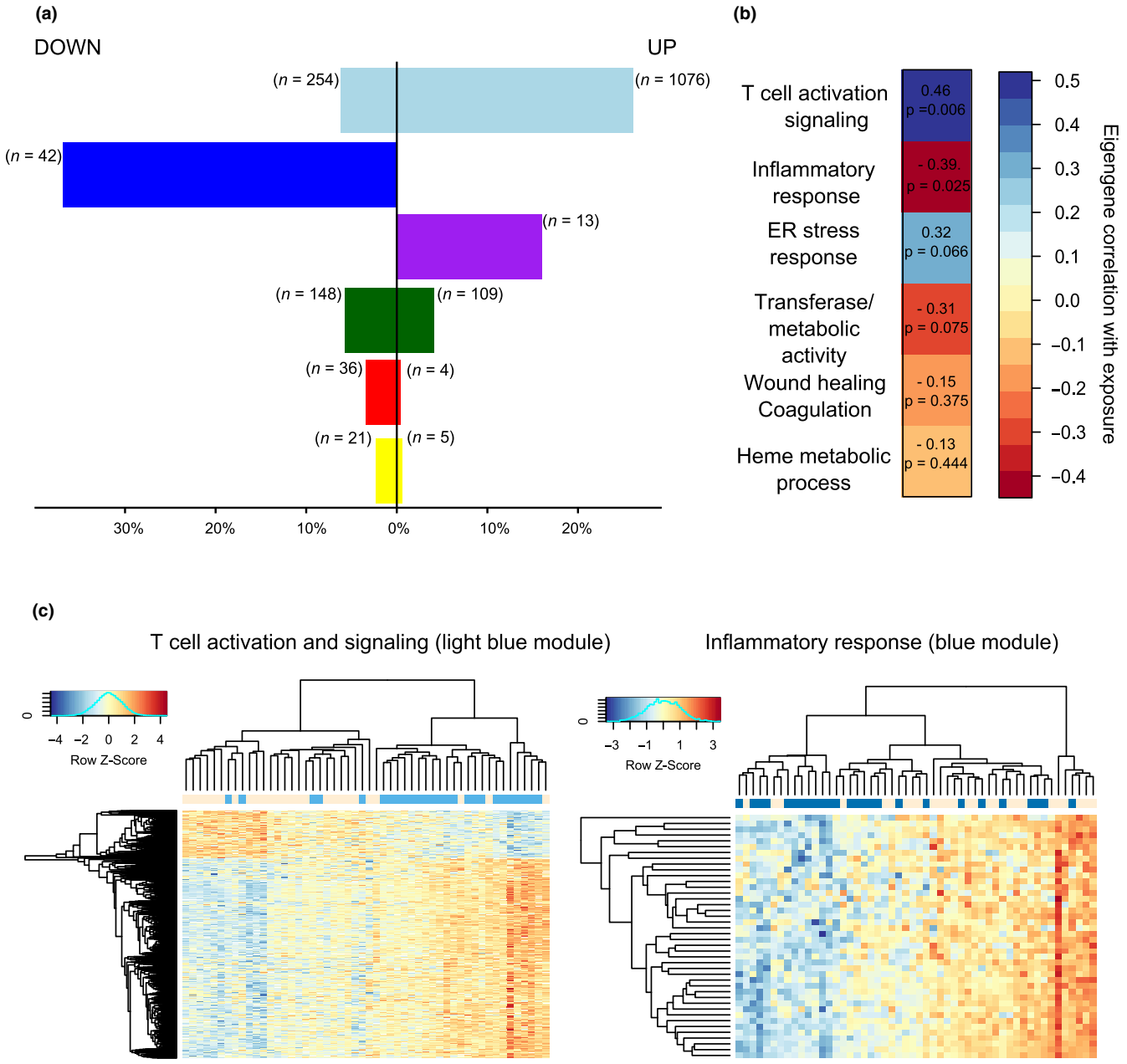

FIGURE 4 (a) Number of significant genes (from linear model) assigned to one of six functional categories (from weighted gene correlation network analysis [WGCNA]) as a proportion of total module size. (b) Correlation between anticoagulant rodenticides (AR) exposure and WGCNA module eigengenes. (c) Heat maps displaying the expression profiles and dendrograms of AR-negative (light colour) and AR-positive (dark colour) bobcats for the "T-cell signalling" and "inflammatory response" modules. Columns are individual bobcats and rows are individual genes [Colour figure can be viewed at wileyonlinelibrary.com]

purple module (Table S7). Similarly, all our modules showed high preservation, with Z.summary scores ranging from 19 to 56 (Table S7).

4 | DISCUSSION

The analysis of genome-wide transcriptional changes is a potent but largely underutilized method to assess organismal response to sublethal toxicant exposure in the wild, especially when controlled exposure experiments are logistically or ethically unfeasible, as is often the case with wild carnivores. Bobcats in the Santa Monica Mountains persistently exposed to ARs do not exhibit canonical signs of coagulation disruption, such as haemorrhaging, despite the fact that this was the second-leading cause of mortality in a long-term coyote study (Gehrt, Riley, & Cypher, 2010). However, bobcats do appear more susceptible to notoedric mange (Riley et al., 2007; Serieys, Armenta et al., 2015), consistent with sublethal effects of AR exposure.

Other environmental toxicants or stressors that potentially influence gene expression may be common in areas where ARs are

deployed. Consequently, ARs may not be the ultimate cause of the pattern we observe or may be one of several contributing factors. However, we argue that ARs are the most likely cause of gene expression dysregulation for the following reasons: (i) ARs are known to accumulate in food chains and are targeted at prey species which bobcats frequently consume (Riley et al., 2010), so there is a specific and well-understood pathway of exposure for bobcats; (ii) AR exposure is correlated generally with more intensive human land use, however AR exposure has also been documented in pristine environments (Gabriel et al., 2012), and particularly near modified open space areas such as landscaped parks, cemeteries, equestrian facilities and golf courses (Nogeire et al., 2015; Serieys, Armenta et al., 2015) which are less degraded than more intensively urbanized settings; (iii) the most urban-associated bobcats in our study area were nonetheless largely using natural areas, with commonly more than 75% or more of their radio telemetry (Riley et al., 2010); (iv) necropsies performed on bobcats throughout the course of the 20+ year study of carnivores in SMMNRA have not shown any other toxicants consistently linked to disease or mortality other than ARs in bobcats or in other carnivores such as coyotes or mountain lions (Beier, Riley, & Sauvajot, 2010; Gehrt et al., 2010); and (v) many of the pathways we have found differentially expressed are known to be affected by ARs as discussed below. For these reasons, we suggest that sublethal AR exposure in bobcats is the best candidate for gene dysregulation and physiologic perturbation.

In addition to impacts related to haemostasis and vitamin K availability, we observed substantial effects on multiple biological processes including xenobiotic metabolism and ER stress response, inflammatory and allergic immune response, adaptive immunity and skin integrity (Figure 2; Table 3). For each process discussed below, these effects have important implications for bobcat health and, taken together, also constitute strong plausible links between AR exposure and mange susceptibility in bobcats.

4.1 | Blood haemostasis and vitamin K

Bobcats, like domestic cats, appear less sensitive than other species to the common effects of ARs (Petterino & Paolo, 2001; van Beusekom, 2015). Specifically, clotting times do not differ significantly between AR-positive and AR-negative bobcats (Serieys et al., 2018). Importantly, however, one bobcat and three mountain lions (Riley et al., 2007) have died from coagulopathy in the study area. Our gene expression results also suggest that there are some direct effects of ARs on haemostasis, potentially related to the vitamin K cycle. We observed GO enrichment for haemostasis-related terms in downregulated genes, and several downregulated genes overlapped with the coagulation module from WGCNA, including genes involved in platelet activation (i.e., thromboxane A synthase 1; *TBXAS1*) and fibrin-clot formation (i.e., serpin family E member 2; *SERPINE2*). Notably, upregulation of *UBIAD1* in AR-positive animals may reflect a possible compensatory mechanism in bobcats. Vitamin K2 has been shown to offset effects of vitamin K antagonists on arterial calcification (Kawashima et al., 1997) and is supportive for hematopoietic and bone metabolism (Miyazawa & Aizawa, 2004; Tabb et al., 2003).

4.2 | Xenobiotic metabolism and endoplasmic reticulum stress

Xenobiotic metabolism is a primary function of the liver that occurs over three phases- cellular uptake, transformation and excretion

TABLE 3 Summary of physiological pathways and processes affected, analytical support, relevant genes of interest and the implications for fitness in anticoagulant rodenticide-exposed bobcats

		Methods				
Pathway/process affected	Pattern	Linear Model	Transcript origin analysis/ transcriptome representation analysis	Weighted gene correlation network analysis	Candidate genes	Implication for fitness
Innate Immunity	↓	↓ Inflammation	↓ total and naïve monocytes	↓ Inflammation	FCER1A, KLF5, KLF 4, GATA2, CPA3, HDC, MS4A2	Decreased defence against extracellular pathogens and allergens
Adaptive Immunity	↑↓	↑ T-cell activation	↑ T- & B-cell activation; ↓ mature/ plasma B cells	↑ T-cell signalling	CD3D, CD3G, CD3E	Immune activation leading to exhaustion; reduced specific antibody
Xenobiotic Metabolism and ER stress	↑	↑ drug metabolism genes	–	↑ ER stress	HYOU1, LAMP3, HSP90B1, XBP1, PDIA6	Increased cell death
Epithelial integrity and wound healing	↓	↓ keratinocyte proliferation	–	↓ wound healing	SFN, IL36B, TGM1, MMP1, MMP10	Reduced epithelial integrity; Increased vulnerability to ectoparasites
Haemostasis and vitamin K	↓	↓ platelet aggregation	–	↓ coagulation	SERPINE2, TBXAS1	Coagulopathy; haemorrhaging

(Filser, 2008; Ioannides, 2001; Lee et al., 2011). During the second phase, reactive intermediates can be formed that directly target enzymes in the ER, thereby triggering oxidative and ER stress responses (Cribb, Peyrou, Muruganandan, & Schneider, 2005; Foufelle & Fromenty, 2016). In bobcats, evidence that AR exposure activates the ER stress response is, as shown by the differential expression of genes such as lysosomal-associated membrane protein 3 (*LAMP3*), heat shock proteins (*HSP90B1*), hypoxia upregulated 1 (*HYOU1*), X box binding protein 1 (*XBP1*) and protein disulphide isomerase (*PDI6*), all of which were clustered in the WGCNA module related to ER stress (Figure 4a,b).

In model organisms, ARs are processed through canonical xenobiotic pathways and are recognized inducers of oxidative stress (Miller et al., 2009; Ware et al., 2015). However, in felids, mechanisms of xenobiotic metabolism are poorly understood (van Beusekom, 2015). For instance, cats are deficient in several enzymes identified as necessary for drug elimination in rats and humans (Court, 2013; van Beusekom, 2015). Similarly, the mammalian gene encoding for UGT1A6, specifically involved in warfarin metabolism, is a pseudogene in the felid family and is therefore not expressed as a functional protein (Shrestha et al., 2011). High tolerance for ARs suggests that felids have possibly developed alternate and perhaps more efficient mechanisms for metabolizing these toxicants. We observed upregulation of *CYP2U1*, a member of the CYP450 gene family whose products are the primary mediators of xenobiotic metabolism (Karlgren, Miura, & Ingelman-Sundberg, 2005; Lynch & Price, 2007; Zanger & Schwab, 2013). In humans, variants in certain CYP enzymes are associated with differential warfarin sensitivity (Freeman, Zehnbauer, McGrath, Borecki, & Buchman, 2000). Given the high variability of CYP function across species (Zanger & Schwab, 2013), it is plausible that *CYP2U1* plays an active role in the metabolism of ARs in felids. Additionally, we observed upregulation of *CHST2* and *HS3ST1*, two genes involved in the xenobiotic metabolism pathway (Zhu, Qu, Xia, Sun, & Chen, 2016), as well as *ABCB1*, essential for elimination of AR metabolites (Miller et al., 2009; van Beusekom, 2015) and also associated with differential warfarin sensitivity (Wadelius et al., 2004).

4.3 | Immunomodulation by ARs

Controlled experiments on herbicides and pesticides document exposure-related changes in circulating leucocyte composition in a variety of species (Cimino-Reale et al., 2008; Malik & Chughtai, 2003). For ARs specifically, rats exhibited reduced monocytes and increased lymphocyte numbers (Mikhail & Abdel-Hamid, 2007). We found evidence of similar patterns of AR-induced changes in circulating leucocytes in bobcats, likely resulting in both immune suppression (of myeloid lineage immune cell function) and stimulation (of lymphoid lineage cell functions).

With respect to immune suppression, we observed downregulation of several genes involved in the allergic immune response including *FCER1A*, *HDC*, *MS4A2* and *CPA3*, each primarily associated with the function of mast cells and monocytes. Evidence of reduced total monocytes in AR-exposed bobcats, with a higher relative abundance of activated or mature to naive monocytes, suggests a decrease in the production of immature myeloid lineage cells. In mammals, white blood cell production (hematopoiesis) occurs in bone marrow, where transcriptional regulation, cytokine signalling and properties of the stromal niche operate in tandem to determine lineage commitment of hematopoietic stem cells (Dorshkind, 1990; Orkin & Zon, 2008; Schoeters, Vander Plaetse, Leppens, & Van Den Heuvel, 1995). We observed downregulation of several transcription factors involved in hematopoiesis in bone marrow. GATA-2 is critical for the production and maintenance of early hematopoietic progenitors (Tsai & Orkin, 1997). Mutations in this gene are associated with myeloid cell abnormalities in humans (Hsu et al., 2011; Pasquet et al., 2013). Transcription factors *KLF4* and *KLF5* share coregulatory roles during hematopoiesis (Ishikawa et al., 2013) including monocyte production and development (Park, Shen, Lewis, & Lacorazza, 2016; Shahrin, Diakiw, Dent, Brown, & D'Andrea, 2016). Further, vitamin K has been shown to improve the supportive function of bone marrow stromal cells for hematopoiesis (Miyazawa & Aizawa, 2004) and directly promotes survival and differentiation of myeloid progenitor cells (Sada et al., 2010). Therefore, AR exposure may impact the number of circulating monocytes through effects of vitamin K availability on bone marrow integrity as well as through deregulation of transcription factors necessary for monocyte differentiation.

With respect to immune stimulation, we observed an increase in gene expression by B- and T lymphocytes in AR-positive bobcats. In B cells, upregulation stemmed specifically from increased activity of naive relative to mature or differentiated B cells. There was also a strong signal for a reduction in the proportion of plasma cells. As above, this may indicate altered output of early lymphocyte progenitor cells, hence inflating the number of naive B cells in peripheral leucocytes. Conversely, it may indicate an increased elimination of standing activated and memory B cells, with a responding increase in lymphopoiesis. In this respect, *KLF5* emerges as an important candidate gene. In heterozygote deficient mice (KLF+/−) this gene has been linked experimentally to the manifestation of systemic sclerosis (SSc) symptoms, a disease characterized by B-cell dysregulation, skin lesions and vasculopathy (Noda et al., 2014). Total and relative naïve B cells were elevated in SSc patients, whereas proportions of memory B and plasma cells were decreased, which was attributable to increased spontaneous death of these cells (Sato, Fujimoto, Hasegawa, & Takehara, 2004). Our results imply that although total B cells are elevated in exposed bobcats, the animal's ability to maintain sufficient memory B cells capable of recognizing specific pathogens upon secondary challenge may be compromised. This could limit the immunologic capacity of exposed bobcats to mount a rapid response to a previously encountered pathogen such as notoedric mange.

Our results also indicate that AR exposure is associated with upregulation of T-cell activity. Indeed, all three of the mature T-cell coreceptor molecules (*CD3G*, *CD3D* and *CD3E*) are highly upregulated in exposed bobcats. Previous work demonstrated that T cells can be activated directly by anticoagulants through MHC

presentation (Naisbitt et al., 2005). Phenindione, for instance, is a vitamin K antagonist anticoagulant that is known to cause hypersensitivity in some human patients. It is also one of the most commonly detected AR compounds (in the form of diphacinone) in our study population (Serieys, Armenta et al., 2015). Manifestation of hypersensitivity occurs primarily in the skin and is correlated with rapid proliferation of drug-specific CD4+ T-cell clones (Naisbitt et al., 2005). In the latter study, it was shown that warfarin (a coumarin compound) can also adopt a phenindione-like structure and similarly elicit T-cell proliferation. Hence, AR exposure may directly induce T-cell proliferation through the antigen presentation, potentially leading to immune exhaustion or expansion of dichotomous (i.e., Th1 and Th2) T-cell subpopulations.

4.4 | Keratinocyte regulation

Genes downregulated in AR-positive bobcats indicated that ARs may interact with epithelial maintenance and formation. Considerable evidence suggests that the skin may be a target tissue of warfarin. Some warfarin-treated patients experienced skin necrosis (Chan, Valenti, Mansfield, & Stansby, 2000; Pourdeyhimi & Bullard, 2014), while endothelial cell injury has been observed in experimental warfarin-treated rats (Ozcan et al., 2012). In bobcats, three differentially expressed genes are consistent with these observations. Transglutaminase 1 (*TGM1*) is a key enzyme in keratinocyte differentiation (Elias et al., 2002; Russell et al., 1995; Thacher & Rice, 1985) and was downregulated in AR-exposed bobcats. Mutations in this gene result in deficient epidermal cornification (Herman et al., 2009) and inhibited skin cell maturation (Jiang et al., 2010). Second, stratifin (*SNF*) is also downregulated in AR-positive bobcats. This gene has been demonstrated to affect the expression levels of matrix metallopeptidases (MMPs) which are integral to the wound healing process (Dong et al., 2008; Medina, Ghaffari, Kilani, & Ghahary, 2007; Nuutila et al., 2012). Interestingly, two metallopeptidases *MMP1* and *MMP10* were some of the most downregulated genes in AR-positive bobcats. Finally, previously discussed transcriptions factors *KFL4* and *KLF5* are involved in epidermal differentiation when expressed in keratinocytes (McConnell, Ghaleb, Nandan, & Yang, 2007; Segre, Bauer, & Fuchs, 1999; Tetreault, Weinblatt, Shaverdashvili, Yang, & Katz, 2016).

4.5 | Potential links between AR exposure and susceptibility to mange

The immune response to mange-causing parasites is highly variable among species (Walton, 2010). With limited understanding of the immunological responses to mange in felids, it is difficult to link mange susceptibility mechanistically to AR exposure in bobcats. One hypothesis based on our results is that simultaneous immune dysregulation and disruption of epithelial integrity specifically predisposes bobcats to opportunistic infection by an ectoparasite pathogen.

Studies of *Sarcoptes scabeii*, a close relative of *Notoedris cati*, indicates that both innate and adaptive immune pathways are activated in response to infestation. In some mammals, an initial localized inflammatory response of the skin, characterized by infiltrates of mast cells, neutrophils and mononuclear cells, is typically followed by a pronounced humoral response, which subsides over time in resistant hosts upon secondary challenge (Arlian, Morgan, Rapp, & Vyszenski-Moher, 1996; Rahman, Lecchi, Fraquelli, Sartorelli, & Ceciliani, 2010). We found that AR-positive bobcats exhibit a substantial reduction in the expression of genes involved in allergic immune response, as well as from both monocytes and late-stage B lymphocytes including plasma cells. Reduction of these cell types in AR-positive bobcats suggests that the basic immune machinery, specifically proinflammatory monocytes, mast cells and antibody-producing B cells/plasma cells, necessary to protect against severe mange infestation, is compromised by ARs. Further, downregulation of proinflammatory cytokines known to operate directly on keratinocytes (e.g., *IL36*) (Foster et al., 2014), in addition to downregulation of several genes involved in epithelial formation and maintenance, 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. Future research should focus on assessing transcriptional changes in skin following AR exposure, as well as determining the impacts on bone marrow integrity and leucocyte production. Further, antibody production against a range of pathogens potentially threatening to bobcats (e.g., Feline Leukaemia virus, Canine Distemper virus, plague, gastrointestinal parasites) should be tested in AR-exposed animals, perhaps in captivity, to assess other secondary effects of AR exposure. In general, experimental models to understand responses to simultaneous toxicant and pathogen exposure need to be developed and tested.

5 | CONCLUSION

We investigated the effects of ARs using RNA-seq and provide convincing evidence that sublethal exposure to ARs has substantial and dramatic gene regulatory consequences in a wild carnivore population. We demonstrate that surveying genome-wide expression from whole blood is an effective method to analyse the effects of toxicants in natural populations. Our analyses provided a system-wide perspective on the physiological effects of these toxicants and enabled us to detect subtle stage-specific changes in circulating leucocyte populations, which has critical implications for the biological function of these cell types. With the increasing accessibility and reduced cost of genome sequencing, this method could be translated to other systems and identify sensitive diagnostic biomarkers for AR exposure in felids and other species. Overall, our results show that the focus on the lethal effects of toxicants developed for pest control which cause a failure of blood to clot in target species may be misplaced. Individual fitness and population persistence may be critically impacted without signs of the target effects of ARs. This result

may apply to other toxicants in the natural environment. Given the worldwide application of anticoagulants in a wide variety of settings from residential to rural environments and even pristine environments, research on the sublethal effects may be a new, previously unacknowledged priority for future research.

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DATA AVAILABILITY

The raw sequencing data, regressed normalized counts, and all associated metadata have been deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession numbers GSE108175 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108175). Tables S1 and S3, as well as HT-seq read counts, GC content and mean gene lengths prior to normalization are available through the DRYAD data repository (https://doi.org/10.5061/dryad.7t7ff).

AUTHOR CONTRIBUTIONS

D.F and L.S. performed the study design. D.F. conducted all RNA processing. A.M. and D.F. performed the majority of analyses and wrote the paper as equal first author contributors. L.S. and S.R. provided samples and laid the foundational premise for the study through previous research. S.C. performed the TOA/TRA analysis and advised in interpretation of immunological results. S.V, M.L. and S.C. performed pathogen screening. R.W. oversaw the research and provided conceptual guidance. All supporting authors provided editorial feedback.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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