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

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26 **Abstract**

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Anticoagulant rodenticides (ARs) are indiscriminate toxicants that threaten non-target 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 leukocyte 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 (ARs); bobcats; gene expression; secondary poisoning

Introduction

Poisons aimed at controlling specific pest species may threaten populations of non-target species. For toxicants that bioaccumulate in the food chain, these threats are greatest to predatory and scavenging species. Although some mortality in non-target 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 et al., 2009; Santadino et al., 2014; Gill & Raine, 2014), and hence, may decrease population viability (Thompson et al., 2014; Rattner et al., 2014; Serieys et al., 2015a).

Anticoagulant rodenticides (ARs) are toxicants used globally to eliminate rodent pests and have been implicated as an important source of mortality in many non-target species that consume poisoned rodents (Eason et al., 2001; Fournier-Chambrillon et al., 2004; Sánchez-Barbudo et al., 2012; Rattner et al.,2014; Dennis et al., 2015; Gabriel et al., 2015; Huang et al., 2016). 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; Elmeros et al., 2011; Christensen et al., 2012). 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

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57 Pesticide Regulation 2013). AR toxicity was a leading cause of mortality in predatory and
58 scavenging birds (Kelly et al., 2014) and in coyotes (*Canis latrans*) (Riley et al., 2003), and it is
59 increasingly recognized as a major threat to the to the Pacific fisher (*Pekania pennanti*) (Gabriel
60 et al., 2012; Thompson et al., 2014) and to the endangered San Joaquin kit fox (*Vulpes macrotis*
61 *mutica*) (Nogeire et al., 2015). In Southern California over 90% of bobcats and mountain lions
62 (*Puma concolor*) tested positive for ARs (Riley et al., 2007). Further, AR exposure occurs in a
63 wide variety of environments, from pristine areas such as the Sierra Nevada Mountains, to
64 agricultural areas with low human densities such as cattle and horse ranches and grain storage
65 facilities, to urban areas with both high and low-density housing, as well as highly modified
66 areas such as golf courses and natural areas which abut human habitation (Gabriel et al., 2012;
67 Gabriel et al., 2015; Nogeire et al., 2015; Serieys et al., 2015).

68 Several formulations of ARs are currently being used and are grouped into first- and
69 second generation ARs (FGARs and SGARs, respectively). The latter are more acutely toxic,
70 requiring only a single feeding in rodents, and are more persistent in tissue as they were
71 developed as a countermeasure to heritable resistance in rodent populations to FGARs. Both
72 categories of AR's have the same molecular target, VKOR (the enzyme that converts vitamin K
73 to the biologically active form), but SGARs typically have a higher affinity for the enzyme, are
74 more resistant to biotransformation, and have a greater bioaccumulation potential (Rattner et al.,
75 2014). The most commonly deployed FGARs are warfarin, chlorophacinone, and diphacinone
76 and the most commonly used SGARs are brodifacoum, bromadiolone, difenacoum, and
77 difethialone (US EPA- https://www.epa.gov/rodenticides/restrictions-rodenticide-products). In
78 the Santa Monica Mountains near Los Angeles, CA (USA), bromadiolone and brodifacoum
79 (SGARs) had the highest prevalence of detection in bobcats, whereas diphacinone (FGAR) was
80 detected at the highest concentrations in animal tissues (Serieys et al. 2015a).

81 ARs are vitamin K antagonists that reduce vitamin K availability for a variety of critical
82 processes including hemostasis, bone metabolism, angiogenesis, apoptosis, oxidative protein
83 folding, and immune function (Opal & Esmon, 2002; Li et al., 2003; Shearer & Newman, 2008;
84 Esmon, 2005; Suttie, 2009; Ferland, 2012; Rutkevich & Williams, 2012; El Asmar et al., 2014;
85 Danziger, 2008). While secondary exposure to ARs frequently leads directly to death from
86 hemorrhaging (California Department of Pesticide Regulation 2013), persistent sublethal

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87 exposure appears to be common in non-target species (Fournier-Chambrillon et al., 2004; Riley
88 et al., 2007; Gabriel et al., 2015; Nogeire et al., 2015). Known side effects of sublethal exposure
89 to vitamin K antagonists in humans and rats include pathologies such as arterial calcification
90 (Danziger et al., 2008), severe skin irritation (Ozcan et al., 2012; Pourdeyhimi et al., 2014) and
91 both immune activation and suppression (Kater et al., 2002; Popov et al., 2013). Given these
92 potential effects, it is likely that sublethal AR exposure in natural populations disrupts important
93 biological pathways necessary for survival from injury and pathogens.

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

104 Despite high exposure prevalence in our study area, few bobcat mortalities have been
105 attributed directly to AR toxicity (Riley et al., 2007). However, previous research repeatedly
106 found mortality from notoedric mange (caused by the mite *Notoedris cati)* to be associated with
107 the level of ARs (Riley et al., 2007; Serieys et al., 2015a), suggesting the potential for sublethal
108 effects of ARs on the ability of bobcats to resist mange mite infection. Mange was the primary
109 source of mortality in the bobcat population from 2002-2008 (Riley et al. 2010, Riley et al.
110 2015), which resulted in a genetic bottleneck (Serieys et al., 2015 b). Notoedric mange had never
111 previously been known to have such severe demographic impacts on any wild felid population,
112 and typically only affected a few individuals that were likely already unhealthy (e.g., Penner and
113 Parke 1954; Pence et al. 1982; Pence et al. 1995). The emergence of this epizootic prompted
114 NPS biologists to submit bobcat carcasses to the California Animal Health and Food Safety
115 Laboratory (CAFHS) for necropsy and full evaluation to assess cause of death and any
116 associated factors. Carcass examination and testing for a panel of eight environmental

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117 contaminants (lead, manganese, iron, mercury, arsenic, zinc, copper and cadmium) in addition to
118 ARs suggested ARs as the only consistent underlying complication in bobcats that succumbed to
119 death from mange infection (Riley et al., *personal communication*). However, the mechanism
120 underlying this potential link between mange and AR exposure remains unknown.

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

129 **MATERIAL AND METHODS**

130 **SAMPLING**

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

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147 and difethialone from tissue, serum or whole blood. Detection of AR exposure in blood can
148 greatly underestimate true exposure prevalence as paired liver samples from necropsied animals
149 frequently tested positive for ARs even in the absence of detection in blood (Serieys et al.,
150 2015a). Several factors may determine the detectability of ARs in blood: time since exposure;
151 the magnitude of exposure; and the metabolic half-life of the AR which is both species and
152 compound specific. Thus, although detection in blood most likely indicates a relatively recent
153 exposure event, we cannot distinguish among all these effector variables. Further, many samples
154 fell below the limit of quantitation but above the level of detection. Hence, we considered AR
155 exposure status as a binary variable (see Serieys et al., 2015a), and conservatively considered
156 individuals showing detectable levels of at least one and up to five of the seven screened
157 compounds (i.e., > 1 ppb) as positive for AR exposure (AR-positive).

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

168 **METHOD DETAILS**

169 ***RNA processing***

170 Total RNA was extracted from 0.5 mL whole blood using the Ambion Mouse RiboPure
171 Blood extraction kit, followed by globin removal using the Ambion GlobinClear Mouse kit (Life
172 Technologies, Inc). RNA was quantified on the Agilent bioanalyzer (Agilent Technologies,
173 USA). RIN scores from globin-depleted RNA samples ranged from 5.5 to 9.3. A minimum of
174 100 ng was used as input for cDNA library preparation using the Kapa Biosystems stranded
175 mRNA kit (Kapa Biosystems, LTD). Each sample was uniquely tagged with custom index
176 sequences developed at UCLA (Faircloth et al., 2014) comparable to Illumina TruSeq tags.

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177 Individual sample libraries were then pooled in equimolar ratios, with 13 or 14 samples per pool
178 and each pool sequenced on two lanes of an Illumina HiSeq 2500 or HiSeq 4000 sequencer
179 (Table S1). Sequencing was performed for 150 bp single end reads. Library quantification,
180 pooling and sequencing were performed at the Vincent Coates Sequencing Facility at UC
181 Berkeley.

182 ***Quality control, mapping and trimming and read quantification***

183 Raw sequences were processed using Trim Galore! 0.3.1 (Krueger, 2015) to remove
184 Illumina adapters and filter out sequences that did not meet the quality thresholds (q > 20, length
185 > 25 bp). Alignment of reads was performed on TOPHAT2 2.1.0 (Kim et al., 2013) using the
186 domestic cat (*Felis catus)* as a reference genome (Ensembl release 85.62) (Yates et al., 2015). To
187 maximize the number of unique reads mapped to the reference genome, we used the following
188 parameters: read mismatches 10, max-insertion-length 12, read-edit-dist 22. On average, 70% of
189 reads mapped uniquely, leaving an average of 13,232,179 mapped reads per individual
190 (3,405,189-22,898,827). Summary statistics are available in Table S1.

191 ***Gene expression quantification***

192 Aligned reads were converted to raw counts using HTSEQ (Anders et al., 2014) with the
193 "union" mode, resulting in alignment to 21,890 genes. After removal of three globin-related
194 genes (ENSFCAG00000030531, ENSFCAG00000031043,ENSFCAG00000022139) with high
195 expression levels prior to normalization, values for the remaining 21,887 genes were normalized
196 using the trimmed mean of M-values (TMM) method in the edgeR package (Robinson &
197 Oshlack, 2010) in R and adjusted for gene length and GC content using custom Python scripts
198 and the package CQN in R (Hansen et al., 2012). The number of genes remaining after filtering
199 for protein-coding genes and sufficient coverage (> 10 reads in 75% of cDNA libraries) was
200 12,332. We used hierarchical clustering of the gene expression adjacency matrix to identify
201 outlier samples (defined as having a z-score greater than 3) with the R package WGCNA
202 (Langfelder & Horvath, 2008).

203 **STATISTICAL ANALYSIS**

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

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205 ***LIMMA***

206 We performed principal components analysis to identify and remove technical factors
207 from the expression data (Figure S2). Gene by gene linear mixed models were used to identify
208 differentially expressed genes in AR-positive bobcats using the limma package in R (Ritchie et
209 al., 2015). We adjusted our significance values to account for multiple hypothesis testing using
210 the false discovery rate (FDR) method as implemented in the qvalue package in R (Storey et al.,
211 2015) and selected genes falling below $Q < 0.05$. We selected the genes falling under a Q-value
212 threshold of 0.05 and then performed Gene Ontology (GO) analysis on the up and downregulated
213 genes that passed this threshold using g:Profiler (Reimand et al., 2016). In g:Profiler (version
214 1682), we used the 12,332 genes as a statistical background and aligned our significant Ensembl
215 gene ID specifically to the *Felis catus* genome. We required a minimum of 2 for the query
216 intersection and applied the Benjamini-Hochberg FDR correction for the significance threshold.
217 The remaining parameters were set using the defaults.

218 ***WGCNA (Weighted Gene Correlation Network Analysis)***

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

227 We first ran a k-means clustering optimization to determine the most likely number of
228 clusters in our expression dataset using the ICGE package in R (Irigoien et al., 2012). In
229 WGCNA, we then followed the automatic, one-step network construction and module detection
230 implemented with the function "blockwiseModules" with an unsigned network algorithm, a
231 power β= 6, corType= bicor, maximum block size = 13000, min module size = 40,
232 mergeCutHeight =0.5, mergingThresh = 0.5. The remaining parameters were kept at the default
233 setting. This cutoff value yielded the "correct" number of modules, including the "grey" module,
234 which contains genes that are not part of any modules. Subsequently, we performed a hub gene

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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 dataset, 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 dataset (reference network) can also be found in another dataset (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.

Transcript Origin Analysis (TOA) & Transcriptome Representation Analysis (TRA)

Transcript Origin Analysis (TOA) was applied as in Cole et al. (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 leukocyte subset-specific expression profiles as reference distributions to generate cell diagnosticity scores for each gene analyzed. The cell diagnosticity scores for AR-associated genes (defined either by fold expression difference (> 1.5) or significance ($q < 0.05$)) 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 leukocyte subsets (i.e., monocytes, dendritic cells, natural killer cells, B lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes,

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265 from GEO data set GSE1133), immature/classical (CD16-) vs mature/non-classical (CD16+)
266 monocytes (GSE25913), M1 vs M2 macrophages (GSE51446), and two data sets comparing
267 naïve B lymphocytes with progressively more differentiated B cell subpopulations (GSE64028
268 and GSE13411).

269 RESULTS

270 *Principal Components of Expression Data*

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

286 *AR exposure as a linear predictor of differentially expressed genes*

287 To identify genes influenced by AR exposure, we used linear regression to measure fold-
288 change (β) and statistical significance (Q). Our dataset included read counts for 12,332 genes that
289 were retained after normalization and low coverage filtering. After applying a false discovery
290 rate (FDR) corrected for multiple testing (Figure S3), a total of 1,783 genes were significantly (Q
291 < 0.05) predicted by exposure status, of which 530 were downregulated and 1,253 were
292 upregulated (Figure 2; Table S3). Eighteen of these genes identified in our model overlap with
293 genes listed in the Comparative Toxicogenomics Database (Davis et al., 2017) as interacting

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294 with warfarin, although the direction of dysregulation was not consistent for all genes with
295 responses observed in rats or humans (Table 1).

296 Downregulated genes were enriched for several gene ontology (GO) terms related to
297 immune function, including response to IL-12 and IL-6; positive regulation of acute
298 inflammatory response; complement-mediated cytotoxicity; myeloid differentiation; monocyte
299 activation; FC-epsilon receptor signaling; and positive regulation of macrophage chemotaxis.
300 Downregulated genes were also enriched for terms related to epithelium including keratinocyte
301 proliferation, glomerulus development, and intestinal epithelial differentiation; and for terms
302 related to vascular processes including Tie-signaling, negative regulation of vasoconstriction,
303 regulation of angiotensin levels in blood, negative regulation of blood circulation, and platelet
304 aggregation. Additional terms related to cell cycle, biosynthetic processes, metabolism,
305 reproductive processes, and transport (Figure 3A; Table S4).

306 We observed downregulation of several genes related directly to wound healing and
307 epithelial integrity, including matrix metallopeptidase 1 (*MMP1*: β = - 0.99; Q =0.038) and
308 matrix metallopeptidase 10 (*MMP10*: β = -1.26; Q =0.01); as well as two important transcription
309 factor involved in white blood cell production and differentiation, GATA binding protein 2
310 (*GATA2:* β = -0.54; Q =0.047) and kruppel-like factor 5 (*KLF5:* β = -0.67; Q =0.016). Further
311 several genes involved in the allergic response were downregulated. These included membrane
312 spanning 4-domains A2 (*MS4A2:* β = -0.79; Q =0.03) and Fc Fragment of IgE Receptor Ia
313 (*FCER1A:* β = -0.88; Q =0.025), encoding for the high affinity IgE beta and alpha receptors, and
314 carboxypeptidase A3 (*CPA3:* β = -1.29; Q =0.019) which is involved in granulocytic mediated
315 inflammation. Bobcats exposed to ARs thus may experience a depressed inflammatory response
316 coupled with diminished epithelial integrity and wound healing response.

317 There were 2.36 times as many upregulated genes, which were enriched for GO terms
318 related predominantly to immune function, specifically to T lymphocytes, as well as terms for
319 gene expression and RNA processing. Immune related terms included positive regulation of
320 immune response, T cell differentiation, thymocyte aggregation, and T cell receptor signaling
321 (Figure 3B; Table S5). Notably, we also observed upregulation of UbiA prenyltransferase
322 domain containing 1 *UBIAD1* (β = 0.38; Q = 0.032), a mammalian gene involved in the
323 biosynthesis of vitamin K2 (Nakagawa et al., 2010; Meehan & Beckwith, 2017), as well as

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324 several genes involved in xenobiotic metabolism including Cytochrome P450 Family 2
325 Subfamily U Member 1 (*CYP2U1*: β = 0.35; Q = 0.016), ATP Binding Cassette Subfamily B
326 Member 1 (*ABCB1*: β = 0.52; Q =0.015), Carbohydrate Sulfotransferase 2 (*CHST2*: β = 0.65; Q
327 = 0.013), and Heparan Sulfate-Glucosamine 3-Sulfotransferase 1 (*HS3ST1*: β = 0.64; Q = 0.039).
328 These results suggest that ARs may activate the adaptive immune system as well as processes
329 associated with xenobiotic metabolism and, potentially, responses to vitamin K deficiency. Other
330 GO terms included gene expression, RNA metabolic process, translation, positive regulation of
331 RNA splicing, response to dsRNA, and ribonucleoprotein complex biogenesis (Figure 3B; Table
332 S5). Several of the genes in these terms relate specifically to immune and cellular stress-
333 responses, likely reflecting increased transcriptional activity due to immune activation and
334 toxicant metabolism.

335 Further, we observed differential expression of several interleukin cytokines (ILs) in AR-
336 positive bobcats (Table S6). Downregulated IL genes were generally regulators of inflammation
337 including *IL13* (β = -0.9; Q = 0.016) and *IL36B* (β = -0.8; Q = 0.013); whereas upregulated IL
338 genes were generally indicators of B and T cell activity, including *ILF2* (β = 0.24; Q = 0.044),
339 *ILF3* (β = 0.25; Q = 0.033) and *IL7R* (β = 0.6; Q = 0.017). Overall, the up- and downregulation
340 of numerous cytokines demonstrate a pronounced dysregulation of critical mediators of immune
341 function, implying both immunosuppressive and stimulating effects of AR exposure.

342 ***Transcript Origin Analysis & Transcriptome Representation Analysis***

343 To identify and quantify cellular subsets that contribute to differential gene expression in
344 AR-positive bobcats, we applied a Transcript Origin Analysis (TOA) and Transcriptome
345 Representation Analysis (TRA). The TOA analyses of major leukocyte subsets showed that AR-
346 downregulated genes originated disproportionately from monocytes (CD14+ cells) whereas
347 upregulated genes originated primarily from helper (CD4+CD8-) and cytotoxic (CD4-CD8+) T
348 cells and CD19+ B cells (Table 2). Further, TRA analyses indicated an average 6.4% reduction
349 in total monocyte prevalence within circulating blood of AR-positive bobcats (mean TRA log2
350 prevalence ratio for monocyte-diagnostic genes = -0.102 ± SE 0.047, $p = 0.039$). These results
351 were consistent regardless of whether the differential expression analysis was assessed by effect
352 size (0.917 fold-change) or as a function of the significance threshold ($Q < 0.05$; 0.952-fold
353 change).

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

365 ***Weighted Gene Co-Expression Network Analysis (WGCNA)***

366 We implemented a WGCNA to assign all 12,332 genes to modules based on patterns of
367 coordinated expression, resulting in 11 modules, including a non-specific module (Table S3)
368 which was consistent with the k-means clustering results (Figure S4). We subsequently assigned
369 each module to functional categories based on GO enrichment analysis of modular hub genes
370 and assessed how many significantly differentially expressed genes (based on the linear model)
371 were assigned to each module (Figure 4A). The dominant expression profile (eigengene) for two
372 of the ten modules were significantly correlated ($p < 0.05$) with exposure after FDR correction
373 (Figure 4B; Figure 4C). Functionally, these modules related to T-cell activation and signaling
374 (Pearson's $r = 0.46$, $p_{adjusted} = 0.006$; light blue module), and the inflammatory response
375 (Pearson's $r = -0.39$, $p_{adjusted} = 0.025$; blue module). In addition, 4 of the remaining 8 modules
376 had an overlap of 10 or more genes that were significant in the linear model. These modules
377 were enriched functionally for transferase activity (green module), wound healing/coagulation
378 (red module), endoplasmic reticulum stress response (purple module), and heme metabolic
379 process (yellow module). Module stability for these 6 modules ranged from 27% - 98%. The hub
380 genes were re-assigned to the original module at 98% for the light blue module, at 88% for the
381 green module, at 96% for the red module, at 78% for the yellow module, at 57% for the blue
382 module, at 27% for the purple module (Table S7). Similarly, all our modules showed high
383 preservation, with Z.summary scores ranging from 19 to 56 (Table S7).

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384 **DISCUSSION**

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

393 Other environmental toxicants or stressors that potentially influence gene expression may
394 be common in areas where ARs are deployed. Consequently, ARs may not be the ultimate cause
395 of the pattern we observe or may be one of several contributing factors. However, we argue that
396 ARs are the most likely cause of gene expression dysregulation for the following reasons: 1)
397 ARs are known to accumulate in food chains and are targeted at prey species which bobcats
398 frequently consume (Riley et al., 2010), so there is a specific and well-understood pathway of
399 exposure for bobcats; 2) AR exposure is correlated generally with more intensive human land
400 use, however AR exposure has also been documented in pristine environments (Gabriel et al.,
401 2012), and particularly near modified open space areas such as landscaped parks, cemeteries,
402 equestrian facilities, and golf courses (Nogieres et al., 2015, Serieys et al., 2015a) which are less
403 degraded than more intensively urbanized settings; 3) the most urban-associated bobcats in our
404 study area were nonetheless largely using natural areas, with commonly more than 75% or more
405 of their radio telemetry (Riley et al., 2010); 4) necropsies performed on bobcats throughout the
406 course of the 20+ year study of carnivores in SMMNRA have not shown any other toxicants
407 consistently linked to disease or mortality other than ARs in bobcats or in other carnivores such
408 as coyotes or mountain lions (Gehrt and Riley 2010, Beier et al. 2010); and 5) many of the
409 pathways we have found differentially expressed are known to be affected by ARs as discussed
410 below. For these reasons, we suggest that sublethal AR-exposure in bobcats is the best candidate
411 for gene dysregulation and physiologic perturbation.

412 In addition to impacts related to hemostasis and vitamin K availability, we observed
413 substantial effects on multiple biological processes including xenobiotic metabolism and ER

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414 stress response, inflammatory and allergic immune response, adaptive immunity, and skin
415 integrity (Figure 2; Table 3). For each process discussed below, these effects have important
416 implications for bobcat health, and taken together, also constitute strong plausible links between
417 AR exposure and mange susceptibility in bobcats.

418 ***Blood Hemostasis and Vitamin K***

419 Bobcats, like domestic cats, appear less sensitive than other species to the common
420 effects of ARs (Petterino & Paolo, 2001; Beusekom, 2015). Specifically, clotting times do not
421 differ significantly between AR-positive and AR-negative bobcats (Serieys et al. *unpublished*
422 *data*). Importantly, however, one bobcat and three mountain lions (Riley et al., 2007) have died
423 from coagulopathy in the study area. Our gene expression results also suggest that there are some
424 direct effects of ARs on hemostasis, potentially related to the vitamin K cycle. We observed GO
425 enrichment for hemostasis-related terms in downregulated genes, and several downregulated
426 genes overlapped with the coagulation module from WGCNA, including genes involved in
427 platelet activation (i.e. thromboxane A synthase 1; *TBXAS1*) and fibrin-clot formation (i.e. serpin
428 family E member 2; *SERPINE2*). Notably, upregulation of *UBIAD1* in AR-positive animals may
429 reflect a possible compensatory mechanism in bobcats. Vitamin K2 has been show to offset
430 effects of vitamin K antagonists on arterial calcification (Kawashima et al., 1997) and is
431 supportive for hematopoietic and bone metabolism (Tabb et al., 2003; Miyazawa & Aizawa,
432 2004).

433 ***Xenobiotic Metabolism and Endoplasmic Reticulum stress***

434 Xenobiotic metabolism is a primary function of the liver that occurs over three phases-
435 cellular uptake, transformation and excretion (Ioannides, 2001; Filser, 2008; Lee et al., 2011).
436 During the second phase, reactive intermediates can be formed that directly target enzymes in the
437 ER, thereby triggering oxidative and ER stress responses (Foufelle & Fromenty, 2016; Cribb,
438 2005). In bobcats, evidence that AR exposure activates the ER stress response is, as shown by
439 the differential expression of genes such as Lysosomal Associated Membrane Protein 3
440 (*LAMP3*), Heat Shock Proteins (*HSP90B1*), Hypoxia Up-Regulated 1 (*HYOU1*), X Box Binding
441 Protein 1 (*XBP1*) and Protein Disulfide Isomerase (*PDI6*), all of which were clustered in the
442 WGCNA module related to ER stress (Figure 4A; Figure 4B).

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443 In model organisms, ARs are processed through canonical xenobiotic pathways and are
444 recognized inducers of oxidative stress (Ware et al., 2015; Miller, 2009). However, in felids,
445 mechanisms of xenobiotic metabolism are poorly understood (Beusekom, 2015). For instance,
446 cats are deficient in several enzymes identified as necessary for drug elimination in rats and
447 humans (Beusekom, 2015; Court, 2013). Similarly, the mammalian gene encoding for UGT1A6,
448 specifically involved in warfarin metabolism, is a pseudogene in the felid family and is therefore
449 not expressed as a functional protein (Shresta et al., 2012). High tolerance for ARs suggest that
450 felids have possibly developed alternate and perhaps more efficient mechanisms for
451 metabolizing these toxicants. We observed upregulation of *CYP2U1*, a member of the CYP450
452 gene family whose products are the primary mediators of xenobiotic metabolism (Zanger &
453 Schwab, 2013; Lynch & Price, 2007; Karlgren et al., 2005). In humans, variants in certain CYP
454 enzymes are associated with differential warfarin sensitivity (Freeman et al., 2000). Given the
455 high variability of CYP function across species (Zanger & Schwab, 2013), it is plausible that
456 *CYP2U1* plays an active role in the metabolism of ARs in felids. Additionally, we observed
457 upregulation of *CHST2* and *HS3ST1*, two genes involved in the xenobiotic metabolism pathway
458 (Zhu et al., 2016), as well as *ABCB1*, essential for elimination of AR metabolites (Miller, 2009;
459 Beusekom, 2015) and also associated with differential warfarin sensitivity (Wadelius et al.,
460 2004).

461 ***Immunomodulation by ARs***

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

469 With respect to immune suppression, we observed downregulation of several genes
470 involved in the allergic immune response including *FCER1A*, *HDC*, *MS4A2*, and *CPA3*, each
471 primarily associated with the function of mast cells and monocytes. Evidence of reduced total
472 monocytes in AR-exposed bobcats, with a higher relative abundance of activated or mature to

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473 naive monocytes suggests a decrease in the production of immature myeloid lineage cells. In
474 mammals, white blood cell production (hematopoiesis) occurs in bone marrow, where
475 transcriptional regulation, cytokine signaling and properties of the stromal niche operate in
476 tandem to determine lineage commitment of hematopoietic stem cells (Dorshkind,1990;
477 Schoeters et al., 1995; Orkin & Zon, 2008). We observed downregulation of several transcription
478 factors involved in hematopoiesis in bone marrow. GATA-2 is critical for the production and
479 maintenance of early hematopoietic progenitors (Tsai et al., 1997). Mutations in this gene are
480 associated with myeloid cell abnormalities in humans (Hsu et al., 2011; Pasquet et al., 2013).
481 Transcription factors *KLF4* and *KLF5* share co-regulatory roles during hematopoiesis (Ishikawa
482 et al., 2013) including monocyte production and development (Park et al., 2016; Shahrin et al.,
483 2016). Further, vitamin K has been shown to improve the supportive function of bone marrow
484 stromal cells for hematopoiesis (Miyazawa & Aiwazawa, 2004) and directly promotes survival
485 and differentiation of myeloid progenitor cells (Sada et al., 2010). Therefore, AR exposure may
486 impact the number of circulating monocytes through effects of vitamin K availability on bone
487 marrow integrity as well as through deregulation of transcription factors necessary for monocyte
488 differentiation.

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

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504 capacity of exposed bobcats to mount a rapid response to a previously encountered pathogen
505 such as notoedric mange.

506 Our results also indicate that AR-exposure is associated with upregulation of T-cell
507 activity. Indeed, all three of the mature T-cell coreceptor molecules (*CD3G*, *CD3D*, and *CD3E*)
508 are highly upregulated in exposed bobcats. Previous work demonstrated that T-cells can be
509 activated directly by anticoagulants through MHC presentation (Naisbitt et al., 2005).
510 Phenindione, for instance, is a vitamin K antagonist anticoagulant that is known to cause
511 hypersensitivity in some human patients. It is also one of the most commonly detected AR
512 compounds (in the form of diphacinone) in our study population (Serieys et al., 2015a).
513 Manifestation of hypersensitivity occurs primarily in the skin and is correlated with rapid
514 proliferation of drug-specific CD4+ T cell clones (Naisbitt et al., 2005). In the latter study, it was
515 shown that warfarin (a coumarin compound) can also adopt a phenindione-like structure and
516 similarly elicit T cell proliferation. Hence, AR exposure may directly induce T cell proliferation
517 through the antigen presentation, potentially leading to immune exhaustion or expansion of
518 dichotomous (i.e. Th1 and Th2) T cell subpopulations.

519 **Keratinocyte Regulation**

520 Genes downregulated in AR-positive bobcats indicated that ARs may interact with
521 epithelial maintenance and formation. Considerable evidence suggests that the skin may be a
522 target tissue of warfarin. Some warfarin treated patients experienced skin necrosis (Chan et al.,
523 2000; Pourdeyhimi et al., 2014), while endothelial cell injury has been observed in experimental
524 warfarin treated rats (Ozcan et al., 2012). In bobcats, three differentially expressed genes are
525 consistent with these observations. Transglutaminase 1 (*TGM1*) is a key enzyme in keratinocyte
526 differentiation (Elias et al., 2002, Thacher & Rice, 1985; Russel et al.,1995) and was
527 downregulated in AR-exposed bobcats. Mutations in this gene result in deficient epidermal
528 cornification (Herman et al., 2009) and inhibited skin cell maturation (Jiang et al., 2010).
529 Second, stratifin (*SNF*) is also downregulated in AR-positive bobcats. This gene been
530 demonstrated to affect the expression levels of matrix metallopeptidases (MMPs) which are
531 integral to the wound healing process (Dong, 2008; Medina et al., 2007; Nuutila et al., 2012).
532 Interestingly, two metallopeptidases *MMP1* and *MMP10* were some of the most downregulated
533 genes in AR-positive bobcats. Finally, previously discussed transcriptions factors *KFL4* and

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534 *KLF5* are involved in epidermal differentiation when expressed in keratinocytes (McConnell et
535 al., 2007; Segre et al., 1999; Tetreault et al., 2016).

536 **Potential links between AR exposure and susceptibility to mange**

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

543 Studies of *Sarcoptes scabeii*, a close relative of *Notoedris cati*, indicates that both innate
544 and adaptive immune pathways are activated in response to infestation. In some mammals, an
545 initial localized inflammatory response of the skin, characterized by infiltrates of mast cells,
546 neutrophils and mononuclear cells, is typically followed by a pronounced humoral response,
547 which subsides over time in resistant hosts upon secondary challenge (Rahman et al., 2010;
548 Arlian et al., 1996). We found that AR-positive bobcats exhibit a substantial reduction in the
549 expression of genes involved in allergic immune response, as well as from both monocytes and
550 late stage B lymphocytes including plasma cells. Reduction of these cell types in AR-positive
551 bobcats suggests that the basic immune machinery, specifically proinflammatory monocytes,
552 mast cells, and antibody producing B-cells/plasma cells, necessary to protect against severe
553 mange infestation is compromised by ARs. Further, downregulation of proinflammatory
554 cytokines known to operate directly on keratinocytes (e.g.. *IL36*) (Foster et al., 2014), in addition
555 to downregulation of several genes involved in epithelial formation and maintenance, suggest
556 that ARs directly affect skin integrity and immunity.

557 We hypothesize that the cumulative effects of these cellular responses to AR exposure
558 increases the susceptibility of individuals to opportunistic parasitism of the skin and inhibits
559 wound healing, allowing for the mange lesions to expand and leading to death. Future research
560 should focus on assessing transcriptional changes in skin following AR exposure, as well as
561 determining the impacts on bone marrow integrity and leukocyte production. Further, antibody
562 production against a range of pathogens potentially threatening to bobcats (e.g., Feline
563 Leukaemia virus, Canine Distemper virus, plague, gastrointestinal parasites) should be tested in

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564 AR exposed animals, perhaps in captivity, to assess other secondary effects of AR exposure. In
565 general, experimental models to understand responses to simultaneous toxicant and pathogen
566 exposure need to be developed and tested.

567 **CONCLUSION**

568 We investigated the effects of anticoagulant rodenticides using RNA-seq and provide
569 convincing evidence that sublethal exposure to ARs has substantial and dramatic gene regulatory
570 consequences in a wild carnivore population. We demonstrate that surveying genome wide
571 expression from whole blood is an effective method to analyze the effects of toxicants in natural
572 populations. Our analyses provided a system wide perspective on the physiological effects of
573 these toxicants and enabled us to detect subtle stage-specific changes in circulating leukocyte
574 populations, which has critical implications for the biological function of these cell types. With
575 the increasing accessibility and reduced cost of genome sequencing, this method could be
576 translated to other systems and identify sensitive diagnostic biomarkers for AR exposure in felids
577 and other species. Overall, our results show that the focus on the lethal effects of toxicants
578 developed for pest control which cause a failure of blood to clot in target species, may be
579 misplaced. Individual fitness and population persistence may be critically impacted without signs
580 of the target effects of ARs. This result may apply to other toxicants in the natural environment.
581 Given the worldwide application of anticoagulants in a wide variety of settings from residential
582 to rural environments and even pristine environments, research on the sublethal effects may be a
583 new, previously unacknowledged priority for future research.

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1033 **DATA AVAILABILITY**

1034 The raw sequencing data, regressed normalized counts, and all associated metadata have been
1035 deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO Series
1036 accession numbers GSE108175
1037 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108175). Supplementary Tables S1
1038 and S3, as well as HT-seq read counts, GC content and mean gene lengths prior to normalization
1039 are available through the DRYAD data repository (doi: 10.5061/dryad.7t7ff).

1040 **AUTHOR CONTRIBUTIONS**

1041 D.F and L.S. performed the study design. D.F conducted all RNA processing. A.M. and D.F.
1042 performed the majority of analyses and wrote the paper as equal first author contributors. L.S.
1043 and S.R. provided samples and laid the foundational premise for the study through previous
1044 research. S. Cole performed the TOA/TRA analysis and advised in interpretation of
1045 immunological results. S.V, M.L and S.Carver performed pathogen screening. R.W. oversaw the
1046 research and provided conceptual guidance. All supporting authors provided editorial feedback.

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1053 **Tables and figures**

1054 Table 1 Differential expressed genes listed as related to warfarin in the Comparative
1055 Toxicogenomic Database[1]

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	↑	0.522664633
adenosylhomocysteinase	*AHCY*	↓	↑	0.182681548
BCL2, apoptosis regulator	*BCL2*	↓ (Vitamin K2 inhibit the interaction and increase expression)	↑	0.497313399
chaperonin containing TCP1 subunit 5	*CCT5*	↑	↑	0.30416539
Eukaryotic Translation Initiation Factor 3 Subunit I	*EIF3I*	↓	↑	0.240188572
Ectonucleotide Phosphodiesterase 1	*ENPP1*	↑	↑	0.835102761
G3BP Stress Granule Assembly Factor 1	*G3BP1*	↑	↑	0.224464977
Heat Shock Protein 90 Alpha Family Class B Member 1	*HSP90AB1*	↓	↑	0.344576673
Heat Shock Protein Family A (Hsp70) Member 8	*HSPA8*	↑	↑	0.340538007
Keratin 18	*KRT18*	↓	↑	0.380455519
NmrA like redox sensor 1	*NMRAL1*	↑	↑	0.384219801

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Nucleobindin 1	*NUCB1*	↑	↑	0.251636364
Proliferation-Associated 2G4	*PA2G4*	↑	↑	0.321882064
Protein Disulfide Isomerase Family A Member 3	*PDIA3*	↑	↑	0.227102201
		↓		
Ribosomal Protein L27	*RPL27*	↑	↑	0.314549077
Selenophosphate Synthetase 1	*SEPHS1*	↑	↑	0.319143095
Tumor Protein P53	*TP53*	Affect the expression	↑	0.336253754
		Increase degradation of TP53 protein		
U2 Small Nuclear RNA Auxiliary Factor 2	*U2AF2*	↑	↑	0.187906376

[1] symbol: ↑ = upregulated, ↓=downregulated

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1057 Table 2 Transcript Origin Analysis for leukocytes and leukocyte subsets[1]

CELL TYPE	*P* value	
	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*

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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*

[1] FD = Fold Change; PBMC = peripheral blood mononuclear cell, N = Number of genes, * = significant

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1062 Table 3 Summary of physiological pathways and processes affected, analytical support, relevant
1063 genes of interest and the implications for fitness in AR exposed bobcats.

Pathway/ process affected	Pattern	Methods			Candidate Genes	Implication for fitness
		Linear Model	TOA/TRA	WGCNA		
Innate Immunity	↓	↓ Inflammation	↓ total and naïve monocytes	↓ Inflammation	FCER1A, KLF5, KLF 4, GATA2, CPA3, HDC, MS4A2	Decreased defense against extracellular pathogens and allergens

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Adaptive Immunity	↑↓	↑ T cell activation	↑ T & B cell activation; ↓ mature/ plasma B cells	↑ T cell signaling	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
Hemostasis and vitamin K	↓	↓ platelet aggregation	-	↓ coagulation	SERPINE2, TBXAS1	Coagulopathy; hemorrhaging

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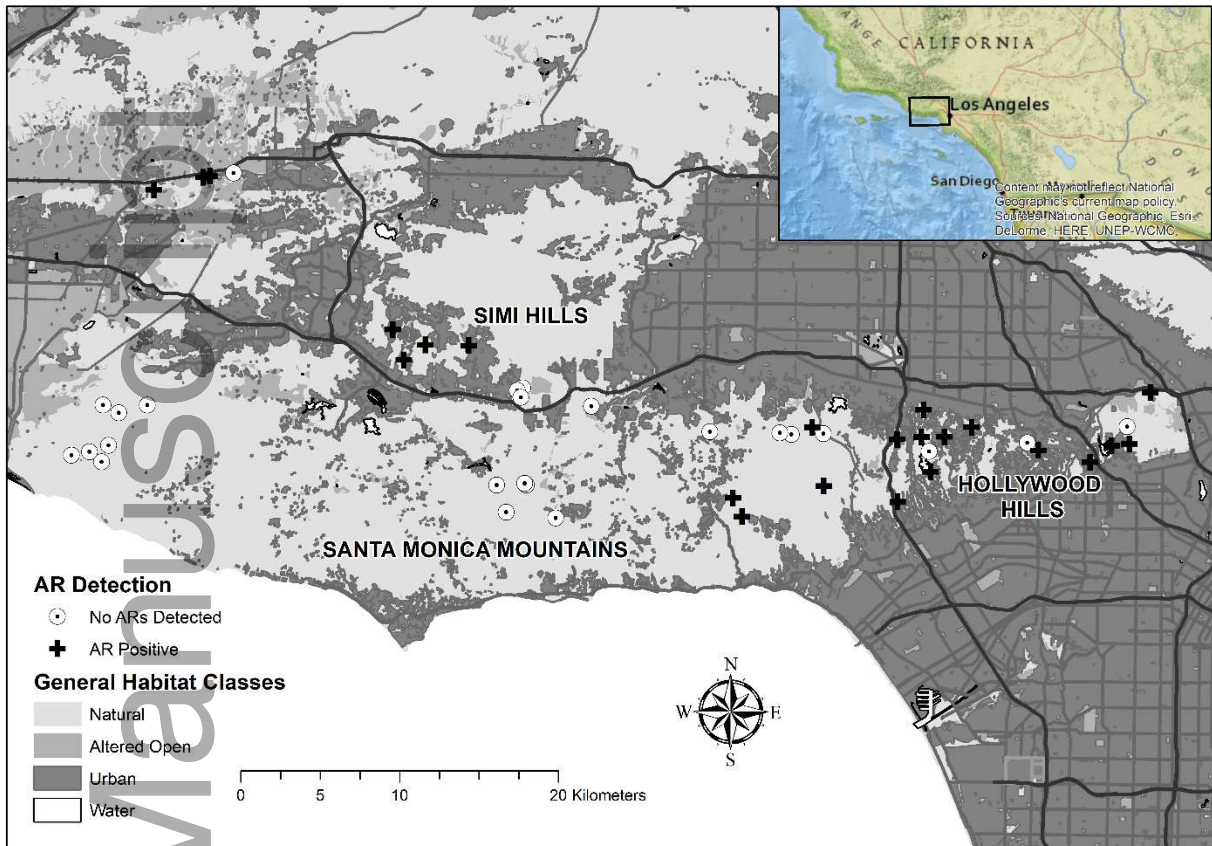

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1066 Figure 1 Map of the study area depicting sample locations for all 52 bobcats, whether or not the
1067 animal tested positive (+) or not positive (⨀) for ARs, and the general land use categories
1068 (urban, altered open, and natural).

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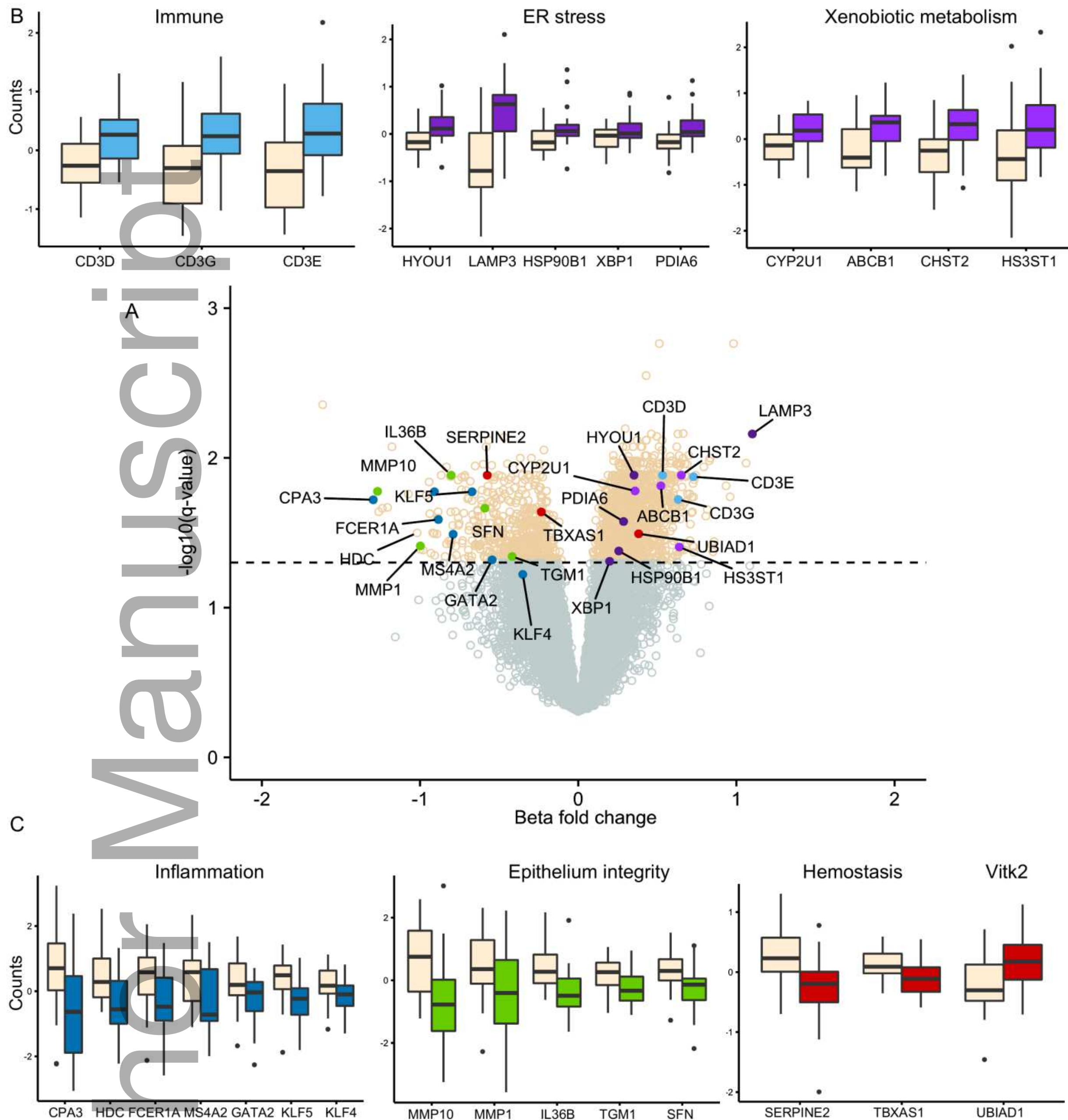

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1072 Figure 2 (A) Volcano plot depicting the -log10 of the Q value against the β fold change for all 12,332
1073 genes. Significant gene (Q <0.05) are highlighted in tan. Labeled genes are color coded by associated
1074 physiological process (depicted in B-C). Mean normalized counts of upregulated genes (B) and
1075 downregulated genes (C) shown for AR-negative (light color) and AR-positive (dark color) bobcats.

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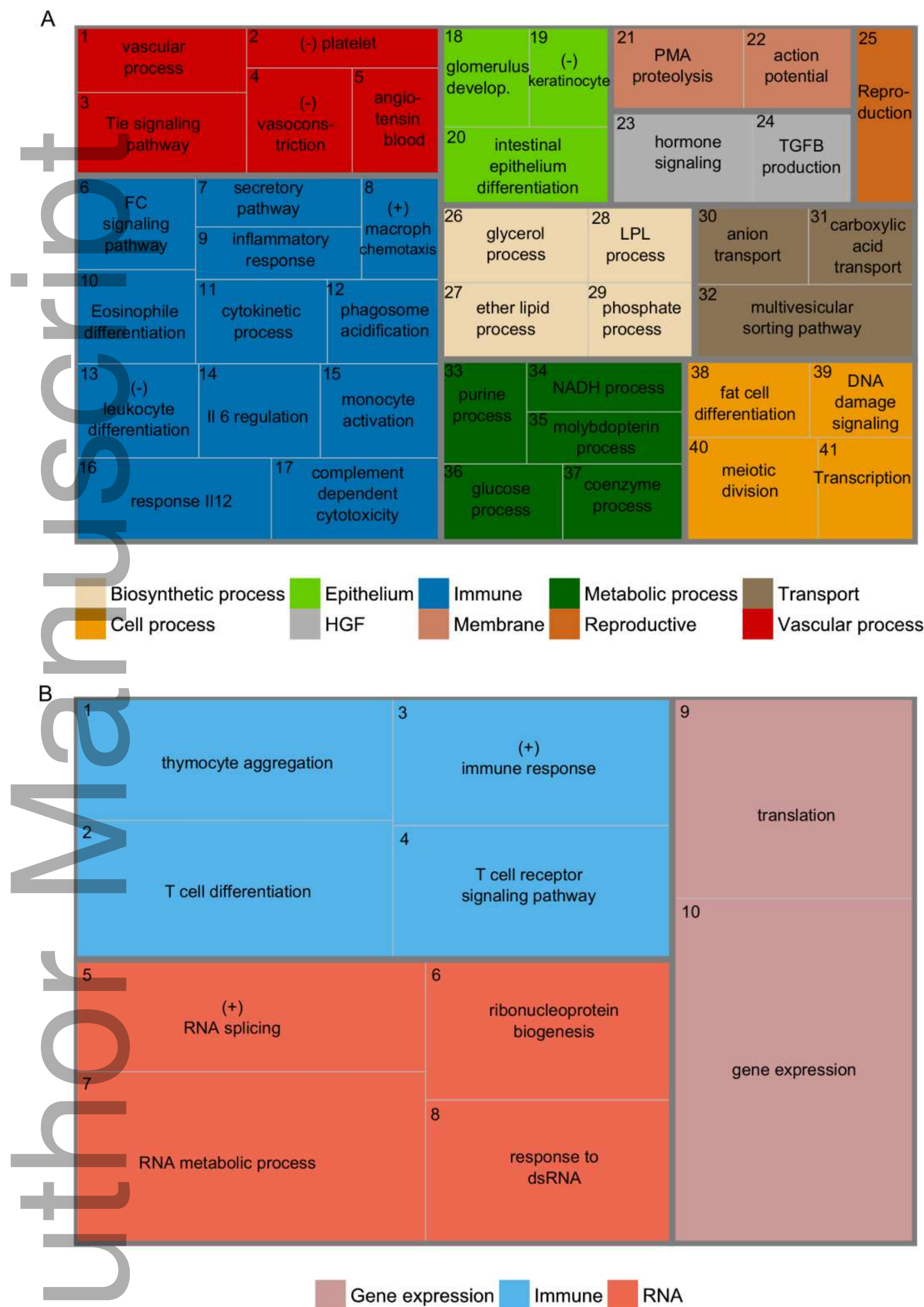

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1076

1077 Figure 3 Treemap of the GO Biological Processes for the down (A) and up (B) regulated genes
1078 (Q <0.05). Box size correlates to the –log10 p-value of the GO-term enrichment. Boxes with the
1079 same color represent higher level categories of processes. Main Abbreviations: (+) : positive
1080 regulation, (-) : negative regulation, macroph: macrophage. See Table S4, S5 for GO term

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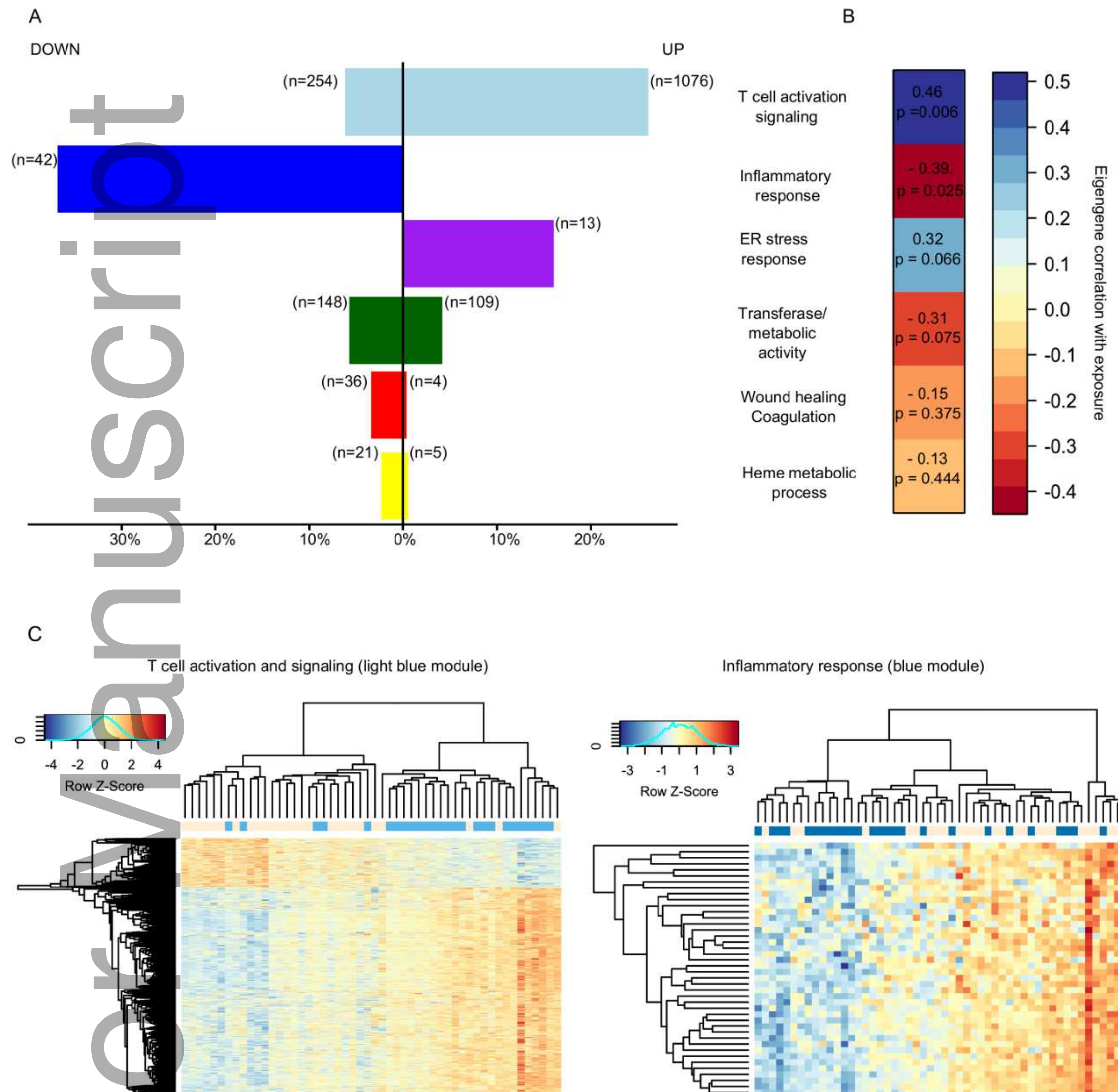
1081 details.



1082

1083 Figure 4 (A) Number of significant genes (from linear model) assigned to one of six functional
1084 categories (from WGCNA) as a proportion of total module size. (B) Correlation between AR
1085 exposure and WGCNA module eigengenes. (C) Heat maps displaying the expression profiles
1086 and dendrograms of AR-negative (light color) and AR-positive (dark color) bobcats for the "T
1087 cell signaling" and "inflammatory response" modules. Columns are individual bobcats and rows
1088 are individual genes.

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Table 1 Differential expressed genes listed as related to warfarin in the Comparative Toxicogenomic Database[1]

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	↑	0.522664633
adenosylhomocysteinase	AHCY	↓	↑	0.182681548
BCL2, apoptosis regulator	BCL2	↓ (Vitamin K2 inhibit the interaction and increase expression)	↑	0.497313399
chaperonin containing TCP1 subunit 5	CCT5	↑	↑	0.30416539
Eukaryotic Translation Initiation Factor 3 Subunit I	EIF3I	↓	↑	0.240188572
Ectonucleotide Phosphodiesterase 1	ENPP1	↑	↑	0.835102761
G3BP Stress Granule Assembly Factor 1	G3BP1	↑	↑	0.224464977
Heat Shock Protein 90 Alpha Family Class B Member 1	HSP90AB1	↓	↑	0.344576673
Heat Shock Protein Family A (Hsp70) Member 8	HSPA8	↑	↑	0.340538007
Keratin 18	KRT18	↓	↑	0.380455519
NmrA like redox sensor 1	NMRAL1	↑	↑	0.384219801
Nucleobindin 1	NUCB1	↑	↑	0.251636364
Proliferation-Associated	PA2G4	↑	↑	0.321882064

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<table>
<tr><td>2G4</td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="2">Protein Disulfide Isomerase Family A Member 3</td><td rowspan="2">PDIA3</td><td>↑</td><td rowspan="2">↑</td><td rowspan="2">0.227102201</td></tr>
<tr><td>↓</td></tr>
<tr><td>Ribosomal Protein L27</td><td>RPL27</td><td>↑</td><td>↑</td><td>0.314549077</td></tr>
<tr><td>Selenophosphate Synthetase 1</td><td>SEPHS1</td><td>↑</td><td>↑</td><td>0.319143095</td></tr>
<tr><td rowspan="2">Tumor Protein P53</td><td rowspan="2">TP53</td><td>Affect the expression</td><td rowspan="2">↑</td><td rowspan="2">0.336253754</td></tr>
<tr><td>Increase degradation of TP53 protein</td></tr>
<tr><td>U2 Small Nuclear RNA Auxiliary Factor 2</td><td>U2AF2</td><td>↑</td><td>↑</td><td>0.187906376</td></tr>
</table>

[1] symbol: ↑ = upregulated, ↓=downregulated

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Table 2 Transcript Origin Analysis for leukocytes and leukocyte subsets[1]

CELL TYPE	P value	
	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*

[1] FD = Fold Change; PBMC = peripheral blood mononuclear cell, N = Number of genes, * = significant

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Table 3 Summary of physiological pathways and processes affected, analytical support, relevant genes of interest and the implications for fitness in AR exposed bobcats.

Pathway/ process affected	Pattern	Methods			Candidate Genes	Implication for fitness
		Linear Model	TOA/TRA	WGCNA		
Innate Immunity	↓	↓ Inflammation	↓ total and naïve monocytes	↓ Inflammation	FCER1A, KLF5, KLF 4, GATA2, CPA3, HDC, MS4A2	Decreased defense against extracellular pathogens and allergens
Adaptive Immunity	↑↓	↑ T cell activation	↑ T & B cell activation; ↓ mature/ plasma B cells	↑ T cell signaling	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
Hemostasis and vitamin K	↓	↓ platelet aggregation	-	↓ coagulation	SERPINE2, TBXAS1	Coagulopathy; hemorrhaging

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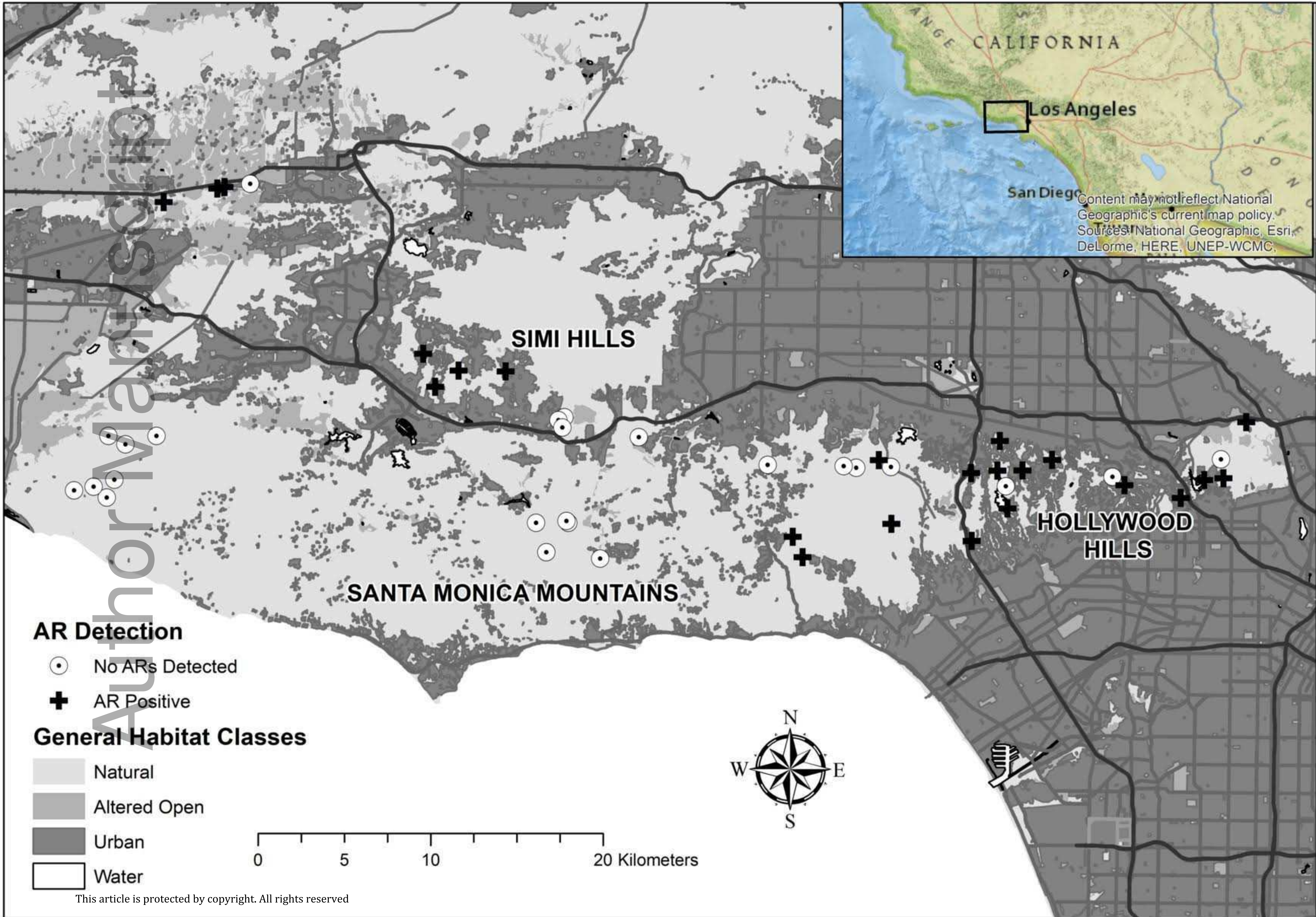

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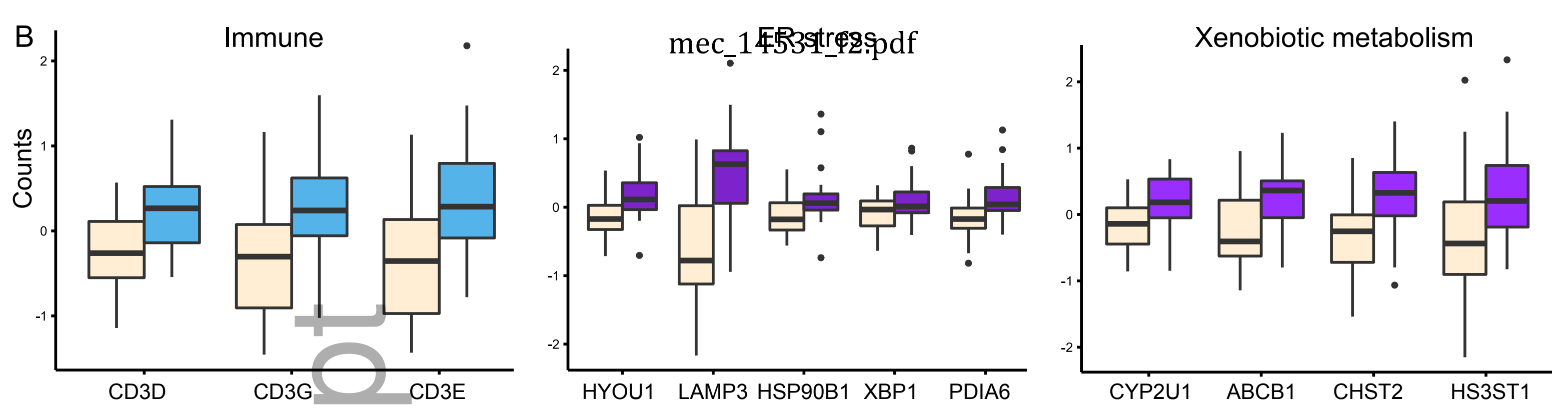

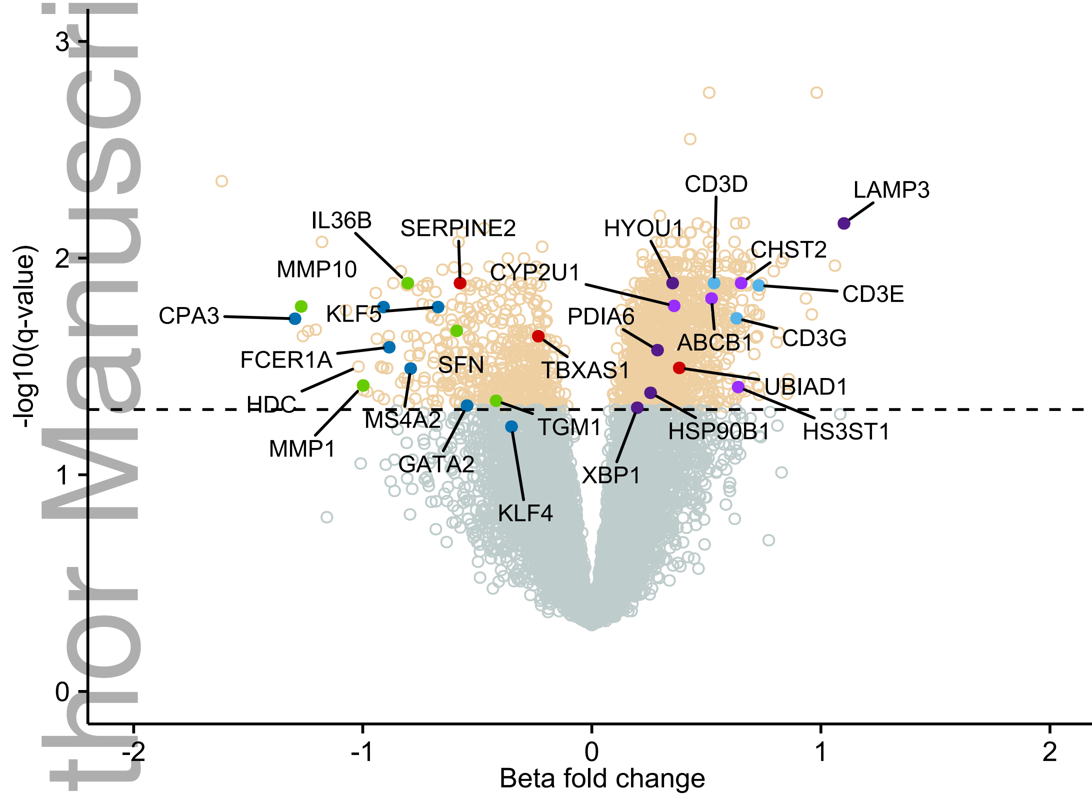

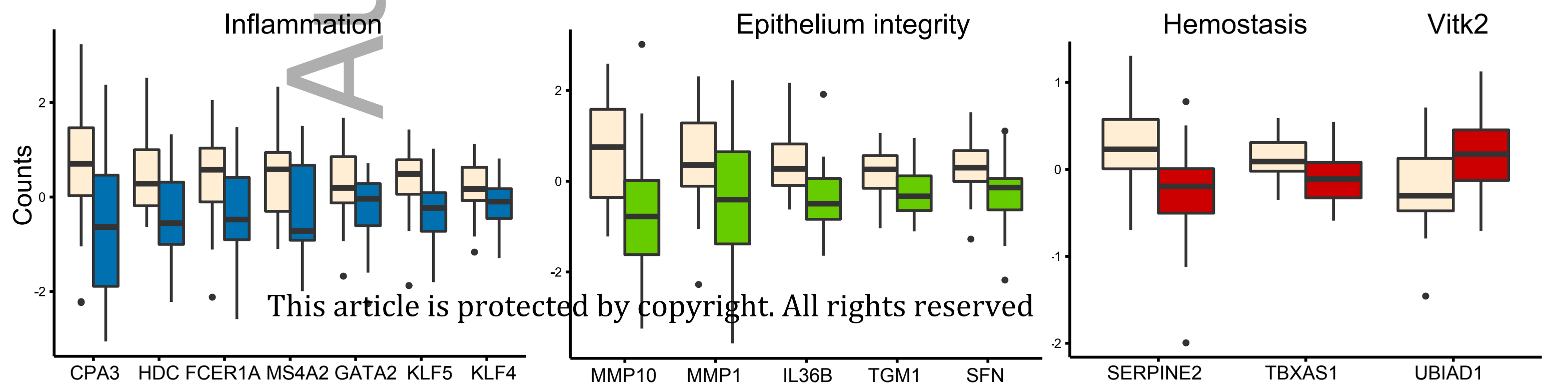

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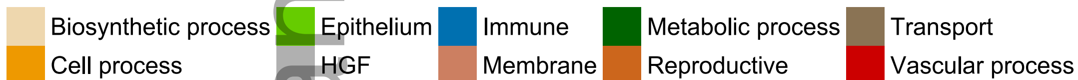
A

1 vascular process
2 (-) platelet
3 Tie signaling pathway
4 (-) vasocons- triction
5 angio- tensin blood
6 FC signaling pathway
7 secretory pathway
8 (+) macroph chemotaxis
9 inflammatory response
10 Eosinophile differentiation
11 cytokinetic process
12 phagosome acidification
13 (-) leukocyte differentiation
14 Il 6 regulation
15 monocyte activation
16 response Il12
17 complement dependent cytotoxicity
18 glomerulus develop.
19 (-) keratinocyte
20 intestinal epithelium differentiation
21 PMA proteolysis
22 action potential
23 hormone signaling
24 TGFB production
25 Repro- duction
26 glycerol process
27 ether lipid process
28 LPL process
29 phosphate process
30 anion transport
31 carboxylic acid transport
32 multivesicular sorting pathway
33 purine process
34 NADH process
35 molybdopterin process
36 glucose process
37 coenzyme process
38 fat cell differentiation
39 DNA damage signaling
40 meiotic division
41 Transcription



B

1 thymocyte aggregation
2 T cell differentiation
3 (+) immune response
4 T cell receptor signaling pathway
5 (+) RNA splicing
6 ribonucleoprotein biogenesis
7 RNA metabolic process
8 response to dsRNA
9 translation
10 gene expression

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A

DOWN

UP

(n=254) (n=1076)

(n=42)

(n=13)

(n=148) (n=109)

(n=36) (n=4)

(n=21) (n=5)

30% 20% 10% 0% 10% 20%

B

T cell activation signaling 0.46 p =0.006

Inflammatory response - 0.39. p = 0.025

ER stress response 0.32 p = 0.066

Transferase/ metabolic activity - 0.31 p = 0.075

Wound healing Coagulation - 0.15 p = 0.375

Heme metabolic process - 0.13 p = 0.444

0.5 0.4 0.3 0.2 0.1 0.0 -0.1 -0.2 -0.3 -0.4

Eigengene correlation with exposure

C

T cell activation and signaling (light blue module)

0

-4 -2 0 2 4

Row Z-Score

Inflammatory response (blue module)

0

-3 -1 1 3

Row Z-Score

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