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Adaptive gene regulation in wild mammals exposed to high predator abundance

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Psychological stress induced by exposure to predators has complex effects on the behaviour and physiology of prey species. This includes potential influences on gene expression mediated via stress-responsive physiological pathways such as the sympathetic nervous system and hypothalamus–pituitary–adrenal (HPA) axis. Laboratory studies have documented diverse transcriptional effects of predator-induced fear, but genomic responses to predator exposure in the wild remain poorly understood. Here, we used RNA-sequencing to investigate the leukocyte transcriptome response to chronic predator pressure in a well-studied population of wild yellow-bellied marmots, *Marmota flaviventer*. We assessed the genomic response to this stressor in three ways by (1) identifying differentially expressed individual genes across the genome, (2) assessing whether differentially expressed genes were statistically over-represented by functional categories and (3) testing for transcription factor activity that may mediate observed gene expression differences. We found 349 individual genes regulated in association with chronic predator presence, including transcripts known to regulate heat shock proteins, metabolism and DNA damage repair. Gene ontology analysis revealed that the majority of these differentially expressed genes were involved with the cellular response to stress, cellular metabolism and protein transport. Transcription factor analysis implicated glucocorticoid signalling in mediating these effects. Our work confirms that the physiological response to predator-induced stress is complex, initiating transcriptional activity in multiple processes and pathways. In addition to the canonical expectations that individuals exposed to predators mobilize HPA signalling and homeostasis pathways, we also detected activity in genes typically associated with human anxiety and cerebral function. This is the first study to demonstrate that leukocyte transcriptomes taken from animals living in a natural environment can reflect the complex ecology of fear.

Psychological stressors can induce dramatic physiological and behavioural responses. Brain-mediated activation of the sympathetic nervous system in response to a threatening stimulus redirects cognitive and physical attention towards the threat. Specifically, heart rate and attention increase, and energy from glucose is mobilized from fat (Sapolsky, Romero, & Munck, 2000). The hypothalamus–pituitary–adrenal (HPA) axis also reacts by releasing glucocorticoid hormones (GCs). These well-studied hormones are key to mobilizing and redirecting stored energy away from long-term investments such as growth and reproduction towards more immediate needs including energy metabolism and locomotor activity (Sapolsky et al., 2000). Vertebrates elevate GCs in response to diverse stressors such as a shortage of food (Clinchy, Zanette, Boonstra, Wingfield, & Smith, 2004; Lynn, Breuner, & Wingfield, 2003), an inhospitable social environment (reviewed by Creel, Dantzer, Goymann, & Rubenstein, 2013) and increased predator exposure and/or abundance (Clinchy et al., 2004; Creel, Christianson, & Schuette, 2013; Van Meter et al., 2009).

These physiological responses are likely to be mediated in part by gene expression. Stress puts cells at risk, and transcriptional mechanisms are critical in preventing cellular damage and neutralizing the effects of various stressors to re-establish homeostasis. For example, genes involved in regulating heat shock proteins alter expression under thermal stress across diverse taxa, including yeast (Causton et al., 2001), mussels (Gracey et al., 2008)

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and guppies (Fraser, Weadick, Janowitz, Rodd, & Hughes, 2011). Pro-inflammatory genes respond to physical stressors such as electrical shock (Blandino et al., 2009; Nguyen et al., 1998), swim tests (Cullinan, Herman, Battaglia, Akil, & Watson, 1995) and exercise (Goebel, Mills, Irwin, & Ziegler, 2000; Walsh et al., 2001). Physical restraint also affects gene expression. Immobilization led to increased inflammatory activity in rat brains (Cullinan et al., 1995; Minami et al., 1991), chicken muscle activated carbohydrate metabolism and cytoskeleton development genes during transport (Hazard et al., 2011) and confined wild canids showed activation of actin and cytoskeleton-related genes (Kennerly et al., 2008). Transcriptional activity thus appears to be a major component in the adaptive response to psychological stress, although patterns vary according to the tissue and stressor examined.

Predation is a powerful selective force and psychological stressor. It has become increasingly apparent that even when predators do not directly kill prey, their presence indirectly affects prey population dynamics, behaviour and physiology (Clinchy, Sheriff, & Zanette, 2013; Martin, 2011; Preisser, Bolnick, & Benard, 2005). Such physiological responses are evident on the molecular level as gene expression consistently responds to predators under experimental conditions. Exposure to a predator (or predator cues) induces activation of a wide range of molecular pathways, including heat shock proteins (Pauwels, Stoks, & De Meester, 2005; Pijanowska & Kloc, 2004), cytoskeleton organization (Pijanowska & Kloc, 2004), inflammation (Su, Xie, Xin, Zhao, & Li, 2011), pathogen defence and visual perception (Sanogo, Hankison, Band, Obregon, & Bell, 2011). However, the transcriptomic response to predators in natural and uncontrolled settings remains poorly understood. To our knowledge, Lavergne, McGowan, Krebs, and Boonstra (2014) conducted the only study examining gene expression associated with predator presence in a wild mammal population. The authors found that brain tissue taken from snowshoe hares, *Lepus americanus*, during periods of relatively high predator abundance (*Lynx canadensis*) showed significantly altered expression of genes involved in metabolism, hormone responses and immune function. This intriguing study revealed for the first time that the molecular response to predation risk can be observed outside of an experimental setting. However, like other stressors, the genomic response to predation threat is likely complex and not uniform across species and tissues. Here we determine whether genes are differentially expressed as a function of predator abundance in blood, a less invasive assay than sampling brain tissue and one that permits larger sample sizes.

Yellow-bellied marmots, *Marmota flaviventer*, in the vicinity of the Rocky Mountain Biological Laboratory (RMBL; Crested Butte, CO, U.S.A.) have been studied continuously since 1962, providing an ideal system to assess the molecular pathways associated with fear of predation. Marmots are prey of several mammalian and avian predators, and predation is a constant threat. In this population, 98% of summer mortality events are due to predation (Van Vuren, 2001), and marmot colonies experience different degrees of exposure to predators. This long-term study has led to significant insights into the direct and indirect effects of predators on this species. Ecologically, the persistence of a marmot colony is better predicted by predation-related attributes such as visibility and underground protection than food-related factors (Blumstein, Ozgul, Yovovich, Van Vuren, & Armitage, 2006). Behaviourally, marmots have evolved a rich repertoire of antipredator tactics to minimize risk. This species can identify potential predators by sight (Blumstein, Ferando, & Stankowich, 2009), sound (Blumstein, Cooley, Winternitz, & Daniel, 2008) and smell (Blumstein, Barrow, & Luterra, 2008), and they communicate predation risk with others using alarm calls (Blumstein, 2007). Glucocorticoid levels are also positively correlated with the degree of predator presence at a colony (Blumstein, Keeley, & Smith, 2016). Thus, marmots in areas of high predator abundance appear to be significantly more stressed compared to those that experience low predation pressure.

To better understand the physiological pathways activated in response to predator-induced stress, we quantified genome-wide transcription levels in blood from yellow-bellied marmots. Whole transcriptome profiling (or RNA-seq) is a valuable tool for assessing cellular physiology because this technique can identify a molecular response to environmental stimuli on many levels, including individual genes, coordinated gene networks and activated regulatory pathways (e.g. transcription factors). We sequenced blood RNA because collection is minimally invasive and unlike more function-specific tissues, it can be used to explore a variety of somatic functions. Leukocytes share approximately 80% of mRNA with other tissues (Liew, Ma, Tang, Zheng, & Dempsey, 2006) and have been used as surrogate for multiple tissues (Davies et al., 2009; Kohane & Valtchinov, 2012; Rudkowska et al., 2011; Sullivan, Fan, & Perou, 2006). Although whole blood is not a perfect surrogate for other tissues, it can provide information in several important pathways, it is responsive to hormones and other systemic influences that impact multiple tissue systems and its availability provides for enhanced statistical power in larger sample sizes.

Our goal was to compare the transcriptomic profile in leukocytes of marmots that experienced high predation pressure with that observed in marmots experiencing low predation pressure as quantified by the frequency of observed predators. We analysed the data at three different levels of cellular response. First, we tested for significant differential expression of individual genes using a genome-wide discovery approach. Second, we tested for enrichment of functional pathways in these genes using gene ontology. Third, we identified upstream transcription control pathways that may mediate these changes in gene expression. Based on previous research examining the transcriptional response to predation (Lavergne et al., 2014; Pauwels et al., 2005; Sanogo et al., 2011; Su et al., 2011), we expected four pathways to be upregulated by individuals experiencing high predator pressure: glucocorticoid signalling, inflammation, metabolism and heat shock proteins.

METHODS

Study Subjects

During the summers of 2013–2015, we studied free-living yellow-bellied marmots in and around RMBL in Gothic, Colorado, U.S.A. Yellow-bellied marmots are facultatively social, sciurid rodents that are active from approximately mid-April to mid-September and hibernate the remainder of the year (Blumstein, Im, Nicodemus, & Zugmeyer, 2004). Marmots were trapped biweekly throughout the active season using Tomahawk live traps and affixed with numbered eartags and unique fur marks to facilitate individual identification from afar (Blumstein, 2013).

Ethical Note

All procedures were approved under Institutional Animal Care and Use Committee (IACUC) research protocol number ARC 2001-191-01 by the University of California Los Angeles on 13 May 2002, and renewed annually, as well as annual permits issued by the Colorado Division of Wildlife (permit number TR-917). In order to not disturb normal marmot behaviour, colony observations were conducted from a distance (20–150 m) using binoculars and 15–45× spotting scopes. We set Tomahawk live traps most mornings and afternoons (at approximately 0700 and 1600 hours Mountain Standard Time), weather permitting. We did not set traps during rain, snow or extreme heat. Marmots were in traps for a

maximum of 2–3 h, and traps were shaded with vegetation on warm days. Marmot handling was brief (typically 5–15 min depending upon the data to be collected). To minimize stress and struggling, handling was conducted with marmots inside a conical cloth handling bag. Carefully trained individuals swabbed the femoral vein with alcohol to reduce the chance of infection, collected up to 3 ml of blood, and applied moderate pressure after venipuncture until bleeding stopped. Marmots were not injured during handling. Individuals were immediately released at the trap location and long-term adverse effects due to trapping procedures were not observed.

Using Predator Abundance as a Proxy for Predator Pressure

Predator presence was calculated using the frequency of daily predator sightings at a marmot colony divided by the number of observation sessions at that colony for each year during 2013–2015. In other words, each day that we observed a colony, we applied a binary score (0 or 1) indicating whether a predator was seen. Species that depredate RMBL marmots include the red fox, *Vulpes vulpes*, coyote, *Canis latrans*, American badger, *Taxidea taxus*, red-tailed hawk, *Buteo jamaicensis*, and golden eagle, *Aquila chrysaetos*. Quantifying predator abundance in this way is a relatively conservative measure because it eliminates the possibility of inflating predator pressure if the same individual predator is observed multiple times in one day. We then divided the number of days predators were observed by the total number of observation days at that colony, for a proportional value ranging from 0 to 1 indicating predator abundance for each colony-year. We limited observations to the early season (mid-April through the end of June) because after this period, vegetation grows rapidly, making terrestrial predators harder to observe. Each year was analysed separately. We then calculated the median predator index across all colony-years. Colony-years with values below the median were considered low predator abundance areas, whereas colony-years above the median were considered high predator abundance areas, as done previously (e.g. Blumstein et al., 2016; Mady & Blumstein, 2017; Monclús, Tiulim, & Blumstein, 2011). All observers were trained to identify both aerial and terrestrial predators at study sites.

RNA Sampling, Library Preparation and Sequencing

We preserved 1 ml whole blood from live-trapped marmots in 2.5 ml PAXgene™ Blood RNA solution (PreAnalytiX, Hombrechtikon, Switzerland). We removed globin transcripts using the rodent GLOBINclear™ kit (Ambion, Waltham, MA, U.S.A.) and assessed RNA quality with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, U.S.A.). To preserve statistical power, we excluded samples with RNA Integrity Number (RIN) < 4 and corrected for RNA degradation by regressing the effect of RIN (a technique validated by Romero, Pai, Tung, & Gilad, 2014, details below). We prepared cDNA libraries using a TruSeq Library Prep Kit v2 (Illumina, Madison, WI, U.S.A.), quantified cDNA with the KAPA SYBR® Fast qPCR library quantification kit (Kapa Biosystems, Wilmington, MA) and pooled 8–10 samples per lane. We used Illumina HiSeq2000 (2013 samples) and HiSeq4000 (2014–2015) platforms at the Vincent Coates Sequencing Laboratory (Berkeley, CA) to create single-end 100 base pair (bp) sequences. We sequenced only yearling marmots to control for any effect of age on gene expression.

Read Mapping

There are a few strategies for analysing gene expression, each with strengths and weaknesses. First, a de novo transcriptome can be built and used to map RNA reads. This technique is excellent for examining transcriptomic divergence between species and for identifying variants such as single nucleotide polymorphisms (SNPs) in RNA-seq data. However, transcriptome assemblies are difficult to resolve unambiguously because variants such as SNPs are often interpreted as multiple isoforms of a gene (Martin & Wang, 2011). Consequently, assemblers perform best when built from a high-coverage RNA library from a single individual whereas assemblies built from multiple individuals require additional mapping to the closest genome for cleaning and annotation. Alternatively, one can map reads to a reference genome of a closely related species. This strategy can result in the loss of a large proportion of RNA reads due to divergence between the reference genome and the RNA sequences, but it performs well in quantifying expression of gene homologues.

The objective of our study was to quantify gene regulation during exposure to an ecological stressor in a relatively large sample of individuals. Therefore, we elected to map reads to the most closely related genome available (thirteen-lined ground squirrel, *Ictidomys tridecemlineatus;* GenBank Assembly ID GCA_000236235.1). We believe this is the most appropriate approach for this study design and have successfully implemented it in a number of similar studies in nonmodel organisms (see Charruau et al., 2016; Fraser et al., 2018; Johnston, Paxton, Moore, Wayne, & Smith, 2016). We removed adapters, short (< 20 bp) and low-quality reads (Phred score < 20) using Trim Galore! (Krueger, 2015) and mapped resulting reads to the thirteen-lined ground squirrel genome using TopHat2 v.2.1.0 (Kim et al., 2013). These species diverged approximately 8.6 million years ago (Bininda-Emonds et al., 2007) and exhibit sequence divergence of 13.2% (Thomas & Martin, 1993). We maximized read mapping by allowing eight mismatches, a 10 bp gap length and a 20 bp edit distance between reads and the reference genome.

Expression Quantification and Outlier Removal

We used HT-Seq's 'union' mode (Anders, Pyl, & Huber, 2015) to quantify uniquely mapped transcripts. Downstream analyses were conducted in R v.3.3.1 (R Core Team, 2016). We acquired HGNC (HUGO Gene Nomenclature Committee; Gray, Yates, Seal, Wright, & Bruford, 2014) gene symbol information for squirrel transcripts in BiomaRt (Smedley et al., 2015). We filtered the data set to include protein-coding genes with at least 10 reads in 75% of libraries and transformed count data for linear modelling. We normalized counts according to sequencing depth, gene length and mean variance using LIMMA's 'voom' function (Law, Chen, Shi, & Smyth, 2014; Ritchie et al., 2015). We created a distance-based network of samples using WGCNA's 'adjacency' function (Langfelder & Horvath, 2008). Samples with connectivity greater than three standard deviations from the mean were considered outliers and removed (as in Horvath, 2011). We also used WGCNA to identify coexpression modules that associate with predator abundance. However, when we ran these analyses, no modules were found to be significantly associated with predator abundance.

Removal of Technical Variation

Batch effects are ubiquitous in high-throughput genetic studies (Leek et al., 2010). To correct this bias, we used principal component analysis (PCA) of the normalized, transformed expression counts to assess variance due to technical factors. We regressed the effect of technical variables that were correlated with any of the first 12 principal components (PCs) using a significance threshold of 0.05.

Genome-wide Discovery Analyses

To identify individual genes that were significantly associated with predator abundance, we created linear mixed models using EMMREML (Akdemir & Godfrey, 2015). Fixed effects included predator pressure (a binary variable; low, high), day of the year and time of day of sample collection (to account for seasonal and circadian variation, respectively) and sex. Kinship was included as a random effect to control for heritability of gene expression (Wright et al., 2014). We calculated kinship based on pairwise relatedness obtained from 12 microsatellite loci using the triadic maximum likelihood approach in COANCESTRY (Wang, 2011). Dependent variables of mixed models were the residuals of the filtered, normalized and regressed gene expression counts. For each gene model, we extracted the *P* value associated with predator pressure and performed multiple hypothesis adjustment using the false discovery rate (*q*; Storey & Tibshirani, 2003) in QVALUE (Storey, Bass, Dabney, & Robinson, 2015). A gene was considered significantly associated with predation when q was < 0.1.

Functional Enrichment Analysis

To identify the biological processes that were statistically over-represented in the differentially expressed genes, we performed gene ontology (GO) analysis using gProfileR (Reimand et al., 2016). We separated up- and downregulated genes and used these two lists as queries in gProfileR. The background list, or null set, of genes included the 9063 detectably expressed (>10 reads in 75% of libraries), annotated protein-coding genes identified in the marmot transcriptome. We set the minimum functional category and intersection sizes to five, used the 'moderate' hierarchical filter and corrected for multiple testing using the 'gSCS' method. The significance threshold was 0.05.

Quantifying Transcription Factor Activity

To evaluate the role of glucocorticoid receptor (GR) signalling in mediating the observed expression differences, we scanned the promoters of all genes showing >1.25-fold difference in expression between colonies with high versus low predator abundance for glucocorticoid response elements using the TELiS database (Cole, Yan, Galic, Arevalo, & Zack, 2005). GR response element prevalence was assessed using the TRANSFAC mat_sim statistic computed over the V$GR_Q6 position-specific weight matrix (Cole et al., 2005). As in previous studies (Miller et al., 2008, 2014), intensity of GR activation was inferred from the ratio of GR response element prevalence within promoters of upregulated genes relative to downregulated genes, with ($\log_2$) ratios averaged over nine parametric variations in promoter length (-300 nucleotides (nt), -600 nt, and -1000 nt to $+200$ nt relative to the RefSeq start site) and response element detection threshold (mat_sim >0.80, 0.90 and 0.95). Statistical significance of mean log ratios was assessed by bootstrap resampling of differentially expressed genes.

RESULTS

Predation Indexes

During the 2013–2015 summer seasons, we observed marmots for 5039 h and detected 300 predators in 27 colony-years. Of these, 184 predators were observed prior to 1 July. These sightings largely consisted of red foxes ($N = 82$), coyotes ($N = 32$) and various raptors ($N = 51$). Predation indexes ranged from 0.013 to 0.463, indicating that a predator was observed nearly every other day in some colonies. The median cut that separated areas of low versus high predator pressure was 0.092 (mean ± SD: low predator index: 0.073 ± 0.036; high predator index: 0.219 ± 0.092).

RNA-seq Samples

We extracted high-quality RNA sequences from 79 individual yearling marmots. We generated 32.5 million reads per individual on average, of which 18.8 million (58.8%) uniquely mapped to the squirrel genome. Of the 22 389 protein-coding genes in the squirrel genome, 11 440 (51.9%) were substantially expressed (≥10 reads in 75% of libraries). We used the 9063 annotated genes in subsequent analyses. Three batch effects significantly influenced one of the first 12 PCs of gene expression ($P < 0.05$): sequencing platform (HiSeq 2000 versus 4000), RNA extraction batch (samples were extracted in seven batches) and input RNA concentration. To control for RNA degradation, we also regressed the effect of RIN (Romero et al., 2014). Clustering analysis revealed one outlier sample, resulting in 78 total individuals for subsequent analyses. Predation indexes for this data set ranged from 0.013 to 0.463 (median = 0.117). Using this median split, 40 RNA samples came from colonies that experienced low predation pressure, whereas 38 RNA samples came from colonies that experienced high predation pressure.

Linear Mixed Effects Models

After controlling for sampling date, time, sex and relatedness, 349 of 9063 annotated genes were differentially expressed as a function of predator index ($q < 0.1$; see Supplementary Material Table S1, for supporting information). Of these, 203 were significantly upregulated (positive $\log_2$ fold changes) in marmots exposed to high predator indexes, whereas 146 were downregulated (negative fold changes; Fig. 1). Upregulated genes included heat shock proteins and genes important for DNA replication and damage repair processes. Downregulated genes included many involved in central nervous system development, anxiety and depression disorders, oxidative damage and toxin exposure.

Enriched Functional Categories

Gene ontology analysis of upregulated genes revealed categorical enrichment of five biological processes (Table 1). In line with our predictions, marmots in colonies with high predator indexes primarily upregulated genes involved in metabolism, protein synthesis and transport, and the cellular response to stress. Downregulated genes were statistically over-represented by one functional category ('metabolic process').

Predation Transcription Factor Analysis

Genes that upregulated >1.25-fold in association with predator pressure showed a significant enrichment of glucocortoid receptor-binding motifs with their promoters (mean ± SD ratio: 2.16-fold ± 0.73, $P = 0.03$). Consequently, marmots that lived in colonies where many predators were observed showed bioinformatic indications of increased glucocorticoid signalling, which is consistent with our hypothesis that these animals are chronically stressed by predators.

DISCUSSION

Even in the absence of direct mortality, the psychological stress induced by predators can have complex and long-lasting effects on prey demography, behaviour and physiology. To our knowledge, this study is the first to reveal that signatures in leukocyte transcriptomes of wild animals reflect the fear associated with predator

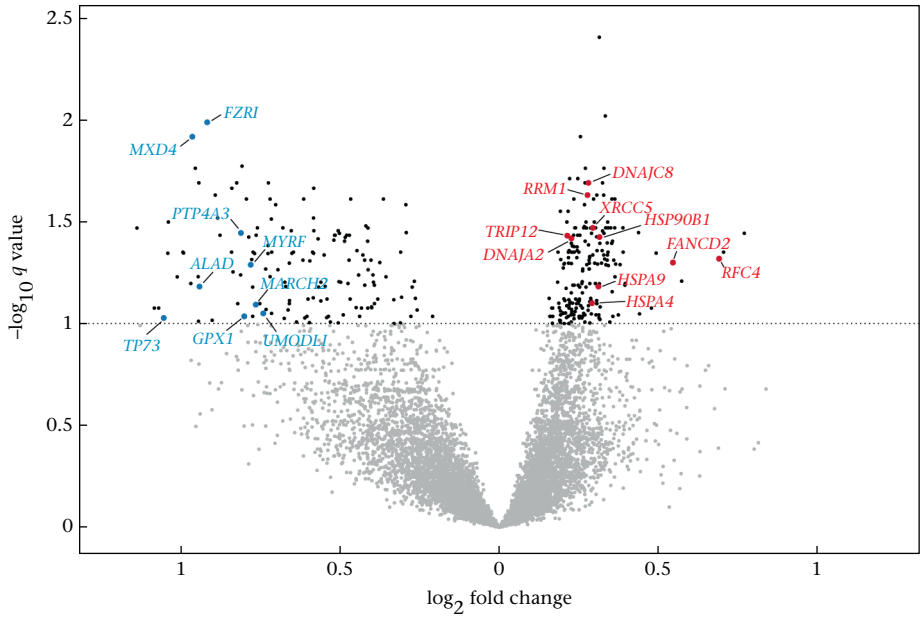

Figure 1. Volcano plot showing fold change in individual gene expression as a function of predator index for 9063 genes. Horizontal dashed line indicates a q value of 0.1. Genes in grey are not significantly associated with predation, black dots are significantly associated ($N = 349$). Genes are highlighted in blue if downregulated and in red if upregulated.

presence. We found that cells taken from yellow-bellied marmots that experienced chronic predator pressure showed differential expression of genes involved in numerous functional pathways and somatic processes, many of which were consistent with previous studies of predator-mediated effects (see below). Based on the extensive associative evidence between inflammation and stress, we were surprised not to have detected the inflammatory transcriptional response we predicted. However, one other observation that did emerge in this study may help explain that lacuna. Our hypotheses regarding glucocorticoid signalling, heat shock protein response and metabolic changes as a function of predator exposure were supported. In addition to their effects on metabolism and other physiological processes, glucocorticoids have potent anti-inflammatory effects (Sapolsky et al., 2000). As such, threat-related activation of glucocorticoid signalling in high predator-exposed marmots may have been sufficient to suppress transcription of pro-inflammatory genes that might otherwise have been activated by less intense forms of stress response (e.g. the pro-inflammatory effects of SNS signalling in the absence of HPA axis activation).

Glucocorticoid Signalling

The HPA axis produces glucocorticoid hormones to modulate energetic reactions to various stressors and restores homeostasis. GCs are among the most commonly used proxies for stress, and measuring levels of these hormones is valuable for evaluating the psychological effects of a stressor, especially in natural systems (Reeder & Kramer, 2005). In fact, previous research in this population has shown that marmots that live in colonies frequently visited by predators have significantly increased faecal glucocorticoid levels (Monclús et al., 2011). We note, however, that this relationship was not statistically significant in this smaller data set (Cohen's $d = 0.05$, $P = 0.39$; Fig. 2).

This study improved our knowledge of GC hormone activity by evaluating the GC transcriptional pathways induced by predators. We predicted that marmots exposed to chronic, high predator abundance would upregulate genes bearing response elements for the glucocorticoid receptor. Transcription factor analysis supported this prediction. We found GC receptor-binding motifs to be statistically enriched in the promoters of genes upregulated during chronic predator exposure. This combination of higher GC hormones and increased GC receptor transcription activity supports our hypothesis that these animals are chronically stressed by repeated exposure to predators.

Heat Shock Proteins

Since their discovery during a severe heat stress experiment (Ritossa, 1962), heat shock proteins (HSPs) have been shown to respond to an array of biotic and abiotic stressors including extreme cold (Matz, Blake, Tatelman, Lavoi, & Holbrook, 1995), desiccation (Hayward, Pinehart, & Denlinger, 2004), disease (Chai, Koppenhafer, Bonini, & Paulson, 1999) and environmental

Table 1
Summary of gene ontology results for genes that were upregulated and downregulated as a function of predator pressure

	Enriched biological process	Number of genes	*P*
Upregulated gene list	Cellular response to stress	28	9.65E-05
	Intracellular protein transport	19	0.00167
	Nucleobase-containing compound metabolic process	85	7.87E-20
	Nucleotide metabolic process	13	0.041
	Ribonucleoprotein complex assembly	8	0.0167
Downregulated gene list	Metabolic process	74	0.000231

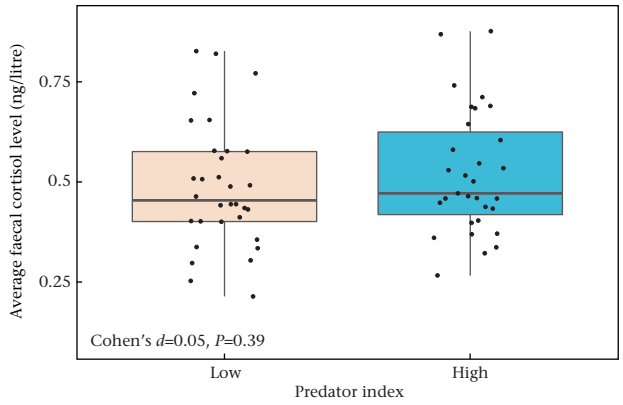

Figure 2. Box plot showing the relationship between predator index and average faecal glucocorticoid measures across the active season. Note that faecal samples were not available for all individuals with RNA sequence data ($N = 63$).

toxicants (Richter et al., 2011). Stressful conditions often result in misfolded proteins and cell death, but HSPs enhance cell survival and maintain homeostasis. HSPs act as molecular chaperones by interacting with other proteins to ensure they are synthesizing, folding and transporting critical proteins correctly during stressful times (Gething & Sambrook, 1992).

Because of this long-standing association between heat shock proteins and the stress response, it was no surprise that five genes that encode HSPs were associated with chronic predator presence in this study. *DNAJC2* (*HSP40* member C2), *DNAJC8* (*HSP40* member C8), *HSPA4* (*HSP70* member 4), *HSPA9* (*HSP70* member 9) and *HSP90B1* were all significantly upregulated by marmots in high-predation colonies (Figs. 1 and 3). Thus, the fear of predator presence was powerful enough to initiate the expression of these important molecular chaperones in marmot leukocytes.

Cellular Homeostasis and DNA Repair

In addition to individual HSPs, we found that the 203 genes upregulated by marmots in areas of high predator presence were statistically enriched for specific homeostatic functions managed by HSPs including the 'cellular response to stress', 'intracellular protein transport' and 'ribonucleoprotein complex assembly' (Table 1). *RFC4, MLH1, RPA2, FANCD2, XRCC5*, *PRKDC*, *TRIP12* and *RRM1* have gene ontology annotations in DNA repair and DNA damage control (Carbon et al., 2017, 2009). Specifically, *RFC4* is a DNA mismatch repair gene that is essential for proper genetic replication and DNA damage checkpoints (Kim & Brill, 2001). *RPA2* binds and stabilizes single-stranded DNA intermediates that form during DNA replication or upon DNA stress (Wold, 1997; Zou & Elledge, 2003). *MARCH2* is a central member of the ubiquitin system (Cheng & Guggino, 2013), which regulates cell homeostasis and appears to be important in the response to thermal and endoplasmic reticulum stress in animals (Verleih et al., 2015; Xia et al., 2017). *MLH1* is a DNA mismatch repair gene that is downregulated during hypoxic stress (Mihaylova et al., 2003). Thus, DNA damage repair appears to be an important component in dealing with the stress associated with chronic exposure to predators in a natural setting as well.

Anxiety-associated Behaviours

The threat of predation is a universally stressful experience. Thus, it is one of the most commonly used stressors in modern studies evaluating anxiety and post-traumatic stress disorders (Cohen, Matar, & Zohar, 2008). Whereas most contemporary studies use laboratory animal models to investigate the transcription behind anxiety behaviours, our results confirmed genetic responses to anxiety in a wild population. We found that *PTP4A3*, which typically exhibits lower expression in humans with major

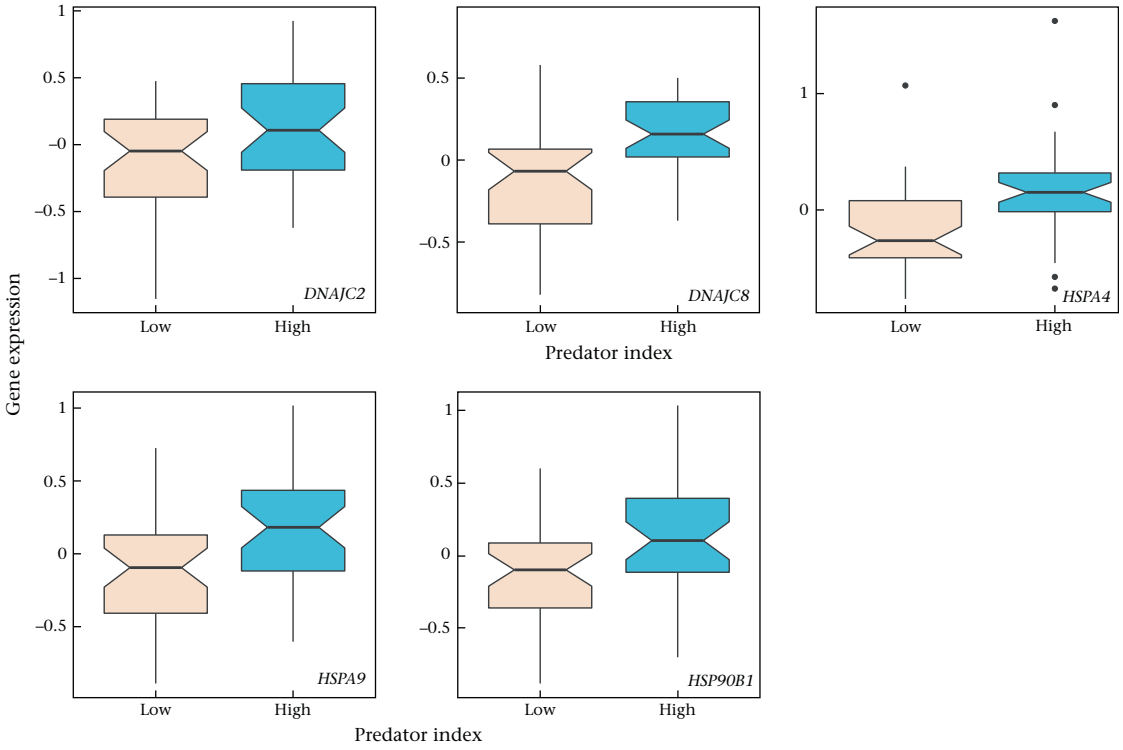

Figure 3. Expression levels of differentially expressed heat shock proteins, grouped by predator abundance index. Each panel represents a single gene, labelled in the lower-right corner.

depressive disorder (Pajer et al., 2012) and post-traumatic stress disorder (Logue et al., 2015), was downregulated in high-predation areas. *ALAD,* a gene associated with social phobias and general anxiety (Donner et al., 2008), and *FZR1*, which is associated with human depression (Tochigi et al., 2008), were also differentially expressed as a function of predator abundance. However, we note that *ALAD* and *FZR1* were previously found to be upregulated by individuals with anxiety disorders, whereas in this data set, they were downregulated by individuals that were often exposed to predators.

We found it a bit surprising that the differentially expressed genes involved in the central nervous response and anxiety disorders (*TP73*, *MYRF, UMODL1, ALAD, FZR1, PTP4A3*) were largely downregulated by marmots experiencing high predator pressure. Although a few of these genes exhibited the same directional fold change as previous studies (e.g. *PTP4A3*), most were observed in the opposite direction (*ALAD, FZR1*). This difference may be due to the chronic nature of this stressor and the species in which we studied these dynamics. Specifically, acute stress is transient and usually provides a protective benefit. Excessive activation of the HPA axis due to chronic stress, however, can lead to pathologies such as sensitization to stressors, impaired hippocampal function and prolonged anxiety-like behaviours (Gray, Rubin, Hunter, & McEwen, 2014; McEwen & Gianaros, 2010). Thus, it is possible that the longer time frame over which these stressors occurred in our study compared to previous studies may have led to sensitization and different transcriptional processes. Furthermore, most of the studies cited above assayed gene expression in humans experiencing social anxiety or depression, whereas we studied wild animals exposed to their predators. An organism's genomic response probably differs dramatically under stressful conditions that threaten survival. Further research is needed to evaluate how these specific genes respond to psychological stressors in different contexts.

Metabolism and Growth

The HPA stress response and GCs mobilize stored energy for immediate needs and suppress long-term growth (Sapolsky et al., 2000). Therefore, we predicted that predator-induced differentially expressed genes would be associated with increased energy metabolism and gluconeogenesis of body proteins.

Since psychological stress is known to promote short-term growth, we specifically expected lipid or carbohydrate metabolic pathways to be enriched in individuals experiencing high predator pressure. However, our data did not confirm this prediction. Gene ontology results indicated an enrichment of activity in metabolic processes, but terms were associated with cellular and nucleotide metabolism, not lipid or carbohydrate metabolism per se. Upregulated genes were largely involved in the 'nucleobase-containing compound metabolic process' and the 'nucleotide metabolic process', whereas downregulated genes were enriched for simply 'metabolic process'.

We did, however, observe differential expression of a few genes that support the stress-induced energy metabolism and growth narrative. *PDK1* encodes for pyruvate dehydrogenase kinase 1, one of the major enzymes responsible for the regulation of homeostasis of carbohydrate fuels in mammals (Mora, Komander, Van Aalten, & Alessi, 2004). *DLD* is also critical in energy metabolism, among its many functions (Dashty, 2013). As predicted, these genes were upregulated by marmots experiencing chronic predator stress. *FADS6* has been implicated in the metabolism of lipids and fatty acids (Guillou, Zadravec, Martin, & Jacobsson, 2010; Liu et al., 2012), but it was downregulated by individuals experiencing high predation abundance. *PHOSPHO1* is involved in the mineralization of bone and cartilage (Houston, Stewart, & Farquharson, 2004). This gene was significantly downregulated by marmots experiencing high predation risk, thus supporting the hypothesis that the chronic stress of predator pressure might suppress skeletal growth.

We acknowledge, however, limitations in our study system and methodological approach. The aim of this study was to examine the transcriptional response to predation stress in a wild, nonmodel organism, which presents both unique insights and unique challenges. At the time of analysis, the closest available genome to the yellow-bellied marmot was the thirteen-lined ground squirrel, which diverged from the study species approximately 8.6 million years ago (Bininda-Emonds et al., 2007). When mapping to distantly related species in this way, sequence divergence between the species can result in reads not aligning to the reference genome or aligning to more than one gene. The former problem can lead to a large amount of missing data whereas the latter may introduce false positive genes and inaccurate interpretations. To prevent such spurious results, we limited analyses to only those reads that mapped uniquely to a single location in the squirrel genome (on average, 59% of reads in each sample mapped uniquely to the reference). However, sequence divergence may still allow marmot reads to align to nonhomologous squirrel genes, potentially resulting in false positive results. Thus, our approach of mapping RNA reads to the squirrel genome may have altered the true list of individual differentially expressed genes associated with fear of predation and, subsequently, the transcription factor and gene ontology interpretations. Careful consideration of methodology and genomic resources should be taken when designing, analysing and interpreting similar RNA-seq studies in nonmodel organisms.

In aggregate, our results suggest that the stress of nonconsumptive predator presence is powerful enough to induce multiple aspects of the cellular stress response in wild prey. We identified individual predator stress-associated genes that transcribe proteins that are critical in maintaining homeostasis and metabolism, found that the majority of these transcripts are involved in the canonical cellular stress response and established that the promoters of upregulated genes were highly enriched for glucocortoid receptor-binding motifs. This transcriptome-wide approach was a unique advance over prior work that selectively focused on a handful of candidate genes or investigated hormonal correlates of stressors. Our analysis confirmed that even in a natural population, cellular transcription of diverse genes and pathways that enhance cellular homeostasis can be observed in response to a powerful psychological stressor.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at https://doi.org/10.1016/j.anbehav.2019.04.008.

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