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SPECIAL TOPIC: ECOPHYSIOLOGICAL EFFECTS OF PREDATION RISK

Impact of high predation risk on genome-wide hippocampal gene expression in snowshoe hares

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Abstract The population dynamics of snowshoe hares (*Lepus americanus*) are fundamental to the ecosystem dynamics of Canada's boreal forest. During the 8- to 11-year population cycle, hare densities can fluctuate up to 40-fold. Predators in this system (lynx, coyotes, great-horned owls) affect population numbers not only through direct mortality but also through sublethal effects. The chronic stress hypothesis posits that high predation risk during the decline severely stresses hares, leading to greater stress responses, heightened ability to mobilize cortisol and energy, and a poorer body condition. These effects may result in, or be mediated by, differential gene expression. We used an oligonucleotide microarray designed for a closely-related species, the European rabbit (*Oryctolagus cuniculus*), to characterize differences in genome-wide hippocampal RNA transcript abundance in wild hares from the Yukon during peak and decline phases of a single cycle. A total of 106 genes were differentially regulated between phases. Array results were validated with quantitative real-time PCR, and mammalian protein sequence similarity was used to infer gene function. In comparison to hares from the peak, decline phase hares showed increased expression of genes involved in metabolic processes and hormone response, and decreased expression of immune response and blood cell formation genes. We found evidence for predation risk effects on the expression of genes whose putative functions correspond with physiological impacts known to be induced by predation risk in snowshoe hares. This study shows, for the first time, a link between changes in demography and alterations in neural RNA transcript abundance in a natural population.

Keywords Heterologous microarray · Hippocampus · Sublethal effects · Chronic stress · 10-year population cycle

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Introduction

The traditional view of predation holds that the primary means by which predators affect prey population dynamics is via direct mortality (Sih et al. 1985). A more complete picture is now emerging, with the emphasis of predator–prey research shifting to also include indirect or sublethal effects, which can have as much, or even greater, impact on prey populations (Preisser et al. 2005; Creel and Christianson 2008). The fear and psychological stress of predator exposure have been associated with many long-term physiological impacts, including alterations in brain morphology, memory and learning, stress hormone levels, and a variety of anxiety-like behaviors (see Clinchy et al. 2011). These impacts may lead to, or be mediated by, changes in gene expression in the brain. Genomic tools can be used to elucidate the molecular mechanisms and gene networks that underlie an organism's ability to detect and respond to changes in their environment, and mount morphological,

behavioral, and physiological responses (Chapman 2001). While laboratory experiments provide compelling evidence for stress-induced neurological effects in mammals (Sapolsky 2003; McEwen 2007), research in natural populations is needed to establish their presence and relevance in natural systems (Calisi and Bentley 2009). It is in nature where such changes would have evolved to adapt organisms to the selective forces acting upon them (MacColl 2011; Boonstra 2013).

One of the major genomic tools available to understand changes in molecular mechanisms in response to experimental treatments is microarrays. Microarrays assess mRNA levels by measuring the binding intensity of target samples to microscopic spots of short complimentary sequences. This allows simultaneous examination of the expression of thousands of genes across experimental conditions. The major hindrance to genomic investigations for wildlife ecologists in the past has been a lack of commercially available technological resources for non-model species (Travers et al. 2007). The design of novel microarrays to examine gene expression depends on gene sequence information that is still often lacking for non-model species (i.e. species that are not mice, rats, or humans). Though new technologies for whole-genome RNA sequencing are developing rapidly, they remain cost-prohibitive for the majority of researchers. These costs can be exacerbated by the larger samples sizes required to achieve adequate power in these populations, given the substantial natural variation and genetic heterogeneity. Microarrays developed for closely related model species have thus been proposed as time- and cost-effective alternatives for investigations in species where little or no genomic resources exist (Eddy and Storey 2008). Heterologous or cross-species arrays have successfully been used to assess experimental and natural variation in gene expression in a variety of species, including wood frogs (Storey 2004), groundhogs (Rinaudo and Gerin 2004), dogs and wild canids (Saetre et al. 2004), naked mole rats (Brodsky et al. 2005), and Weddell seals (Ptitsyn et al. 2010).

Snowshoe hares (*Lepus americanus*) are a keystone herbivore species whose predictable 8- to 11-year population cycle dominates terrestrial community dynamics throughout Canada and Alaska's 5,000,000 sq km boreal forest (Krebs et al. 1995). During the course of a single cycle, hare densities can fluctuate up to 40-fold. The cycle is top–down regulated by direct predation that is key to all but the 2- to 5-year low phase. As the hare population increases, so do their predators (lynx, coyotes, great-horned owls), following a 1- to 2-year lag. Reproductive output in hares is also cyclical, with maximum rates occurring during the early increase phase when predator densities are low, and minimum rates when predators peak during the decline (Cary and Keith 1979; Stefan and Krebs 2001). Several hypotheses have been put forward to explain the extended low phase that follows the decline (see Boonstra et al. 1998a, b), when hare populations fail to recover even though the predators have declined markedly and there is an abundance of high-quality forage available (Krebs et al. 1986; Boutin et al. 1986). Rapid genetic selection over the cycle of the type envisioned by the polymorphic behavior hypothesis (Chitty 1967) is highly unlikely as an explanation (Boonstra et al. 1998b), as hares do not have the spacing behavior that is likely to be affected by population density (Boutin 1984), nor are there strong associations between kin (Burton and Krebs 2003). A large-scale manipulation of experimental food addition, fertilizer addition, and predator removal in the boreal forest of the southwestern Yukon showed that none of these treatments can completely prevent hare population declines (Krebs et al. 1995). Rather, a combination of food addition and protection from predators was the most successful in dampening the effects of the decline, which suggests a three-trophic-level interaction of population regulation in hares.

The Chronic Stress Hypothesis (Boonstra et al. 1998a) postulates that the low phase's population lag is a consequence of the chronic stress effects, impaired immunity, and reduced condition seen in decline phase hares due to the intense predation they experience. During the decline, abundant predators are directly responsible for >86 % of hare deaths, and annual adult survival can drop to 0.5 % (Boutin et al. 1986; Hodges et al. 2001). In addition, high predation risk interacts with food limitation during the peak (Hodges et al. 2006), but not the low (Hodges and Sinclair 2003), to cause mass loss and reduce body condition. These are associated with the reduced fecundity that characterizes the peak and decline phases of the cycle. Monitoring of natural populations in the Yukon found that hares are extremely sensitive to variations in predation risk. Fecal stress hormone concentrations fluctuate in synchrony with predator density among phase years, and are highest during the decline phase when predator numbers peak (Sheriff et al. 2011). Results from a laboratory breeding study suggest that cyclic variation in predator-induced stress results in intrinsically different snowshoe hare population morphs (Sinclair et al. 2003). In this study, wild-caught hares from two sequential population cycles' high- and low-predator phases were brought into captivity and allowed to breed within lineages. Despite breeding under identical ideal conditions (no predators, food ad libitum), both "high-predation" lineages had rapid declines in annual reproductive rates, eventually going extinct, while "low-predation" hares maintained their reproductive capacity (Sinclair et al. 2003). Together, these results illustrate the tremendous potential impact the indirect effects of predation can have, and provide further support for predation as the central organizing process affecting snowshoe hare population

cycle dynamics. Thus far, we have extensive evidence of downstream changes in the stress response of snowshoe hares and those bodily processes impacted by stress (e.g., energy mobilization, immune response, reproductive response, etc.; Boonstra et al. 1998a; Sheriff et al. 2009, 2011). In the present study, we examine upstream changes, i.e. changes at the gene expression level that can mediate downstream impacts.

We use an oligonucleotide microarray designed for a closely related species, the European rabbit (*Oryctolagus cuniculus*), to compare genome-wide gene expression profiles in the brains of snowshoe hares harvested from the southwestern Yukon during 1 year of each of the peak (1998, $n = 10$) and decline (2000, $n = 11$) phases. Population densities of hares and lynx, their main specialist predator, were continuously monitored during this cycle. The hippocampus was selected owing to its critical role in regulating the hypothalamic–pituitary–adrenal (HPA) axis, and its sensitivity to the effects of chronic stress (Sapolsky et al. 2000; deKloet et al. 2005). We test the hypothesis that gene regulation in the hippocampus is altered as a function of variation in predation risk in snowshoe hares. Our aim was to identify candidate genes and regulatory pathways that are differentially regulated based on the phase of the population cycle, and that may play a role in regulating physiological response to predation risk and stress susceptibility. We predict that, as the hare cycle progresses from the peak to the decline phase, the link between predation risk and the resultant physiological signatures (documented in Boonstra et al. 1998a; Sheriff et al. 2011) will be marked by changes in hippocampal gene expression and biological pathways associated with increasingly severe chronic stress.

Materials and methods

Population monitoring

Snowshoe hares have been continuously monitored in the southwestern Yukon Territory since 1976, thus abundance data spanning the course of four consecutive population cycles are available (Krebs et al. 2001; Krebs 2011). To obtain population estimates, we conducted 2–3 night trapping sessions on monitoring grids in March/April and September/October. Each grid had 86 Tomahawk traps (No.106; Tomahawk Live Trap, Tomahawk, WI, USA, http://www.livetrap.com) located along four trap-lines, for an effective grid size of ~60 ha. Live-traps were prebaited with alfalfa cubes for 3–5 days before being set, then baited with alfalfa and snow or apple for moisture during trapping sessions. At peak hare densities, some grids had additional traps to reduce trap saturation (all trapping methods described in Hodges et al. 2001). Mark–recapture population estimates were calculated from the maximum-likelihood spatial estimator in Efford's DENSITY 4.4 program (Efford et al. 2009) to provide absolute density estimates for each capture session. Lynx density estimates were derived from winter track counts (see Sheriff et al. 2011). Predation risk was then inferred as a function of hare and lynx densities, overwinter hare mortality rates, and spring lynx:hare ratios in each of these years (Sheriff et al. 2011).

Collection of animals for brain samples

In both years, hares were collected at the same time of year and in the same manner. Hares were live-trapped using No.106 Tomahawk traps in the Shakwak Trench east of Kluane Lake, Yukon Territory (61°N, 138°W) during the third week of February in peak (1998) and decline (2000) years of the snowshoe hare population cycle (Krebs 2011). Traps were set between 1800 and 2400 hours and checked between 0600 and 1000 hours. Trapping did not occur if the overnight temperature was expected to fall below −20 °C. Upon capture, males were weighed with a Pesola spring scale (±10 g) and their right hind foot (RHF) length was measured as an index of body size. Potential differences in body size and condition were considered as sources of variation between years in our analysis. Hares were euthanized via cardiac injection of T-61 (Hoechst, Munich, Germany). Whole brains were rapidly dissected then placed on a glass plate that was covered in sterile aluminum foil and that rested on a bed of dry ice. After freezing, each brain was double-wrapped in parafilm and then in aluminum foil. Brains were kept on dry ice for transport to the University of Toronto and then stored at −80 °C until analysis. All procedures were approved by the University of Toronto Animal Care Committee in accordance with the guidelines of the Canadian Council for Animal Care.

Brain dissection, RNA extraction, and microarray hybridization

The left hemisphere of the hippocampus was dissected from peak (1998, $n = 10$) and decline (2000, $n = 11$) phase adult males. RNA was extracted from 30 mg of mechanically-homogenized tissue with an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) to minimize DNA contamination, then checked for quantity and quality (Nanodrop/Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturers' instructions. Transcript abundance was quantified by microarray for a subset of 8 males from each year. We sent 500 ng of total RNA ($OD_{260}/OD_{280} \geq 2.0$) from each individual to University Health Network's Microarray Facility (Toronto, ON, Canada) for cDNA conversion, fluorescent labeling,

and array hybridization. To avoid any confounding effect of dye assignment, half of the samples from each year were labeled with cyanine 3-cytosine triphosphate, and half with cyanine 5-cytosine triphosphate. Snowshoe hare cDNA samples were hybridized to Agilent Technologies' Rabbit Gene Expression microarray (020908), as rabbits *(Oryctolagus cuniculus)* are snowshoe hares' most closely related model species. This array includes a total of 43,663 probes corresponding to approximately 12,000 genes. Each dual color array was hybridized with 100 ng of differentially labeled cDNA from both a peak and a decline subject for 17 h at 65 °C and 10 rpm. Arrays were subsequently scanned using an Agilent Technologies High Resolution Microarray Scanner (Santa Clara, CA, USA).

Microarray analysis

Array data were imported into Genespring v11.5.1 (Agilent Technologies) for analysis and a standard spatial detrending normalization for dual color Agilent arrays was applied. The average background expression across both dye channels plus 2 standard deviations was used as a lower boundary for probes showing any measured expression. Only probes that showed a signal above this in ≥25 % of the samples were kept. Data were log-transformed, and significant differences between populations were identified for each probe using standard *t* tests. Multiple testing corrections of the data using strict filtering (Benjamini-Hochberg, $p < 0.05$) yielded no statistically significant results. To increase the power of our analysis, differences in gene expression between years were considered significant when multiple (≥2) independent probes interrogating the same gene on the array showed significant differential hybridization between groups ($p < 0.05$). This more relaxed filtering approach was selected to maximize the detection of subtle but potentially biologically relevant differences between years that could then be validated by quantitative real-time polymerase chain reaction (qPCR).

Quantitative real-time PCR validation

Hippocampal RNA samples from all peak (1998, $n = 10$) and decline (2000, $n = 11$) phase males were used to validate microarray gene expression levels by qPCR using a StepOne Plus (Life Sciences). Six genes showing significant differences in transcript abundance between years on the array were selected for validation. Primer sequences were designed in Primer3 using *O. cuniculus* genomic sequences (UCSC Rabbit Genome Browser, Apr 2009 oryCun2.0 assembly), and were selected for high target specificity and mammalian sequence conservation (Table 1). Real-time PCR reactions were performed using Fast SYBR Green Master Mix: 1 μM forward primer, 1 μM reverse primer, and 1 μl of cDNA. A standard curve was generated from 10 serial dilutions of pooled cDNA from all subjects, and samples were assayed in triplicate. Expression values for six commonly used reference genes were also quantified, and qbase geNorm Plus software (Biogazelle, Belgium) was used to identify the three that were most stably expressed in these populations (PPIA, GAPDH, and YWHAZ; data not shown). A normalization factor was then calculated for each subject based on the geometric mean expression of these three genes (Vandesompele et al. 2002). qPCR data were normalized by taking the ratio of target gene expression values to these normalization factors, and one-tailed *t* tests were used to test for significant differences in gene expression between years.

Functional enrichment analysis

Despite being a frequently used model organism in biomedical research, the rabbit genome is poorly characterized and rabbit sequences are still mostly absent from major biological pathway databases such as KEGG and HomoloGene (Craig et al. 2012). BetterBunny (v.2.1) is an online tool designed to analyze output gene lists for Agilent's rabbit array, using protein sequence similarity to provide orthologous human, rat, and mice genes for any given list of differentially regulated microarray probes (Craig et al. 2012). This approach allowed us to take advantage of those model species' well-characterized gene functional and biological interaction pathways. Homologous gene lists were generated using protein sequences with at least 50 % identity similarity, a threshold above which Sangar et al. (2007) found that the risk of transferring erroneous functional annotation between homologs is less than 6 %, thus the probability of similar function at the gene ontology level is high. The homologous human, rat, and mouse gene lists generated by BetterBunny were exported to the Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.7), and each was analyzed for overrepresented annotation terms. The Functional Annotation Cluster (FAC) tool grouped the enriched terms (related to biological pathway, molecular function, and cellular components) associated with each gene into similar or related groups (Huang et al. 2008). FAC analyses were performed for both up- and down-regulated genes using DAVID's high stringency default classification parameters, which select for fewer clusters but higher gene–gene term similarity thresholds, and therefore stronger associations between genes (Huang et al. 2008). Each cluster is assigned an Enrichment Score (ES), which is calculated as the geometric mean (in –log scale) of individual *p* values associated with each annotation term within the group (Huang et al. 2008). Enrichment Scores greater than 1.3 are equivalent to non-log scale $p < 0.05$. This tool allowed for biological interpretation of

Table 1 Target and reference gene primer sequences, annealing temperatures, and amplicon lengths used for quantitative real-time polymerase chain reaction (qPCR) validation of microarray results for snowshoe hares (*Lepus americanus*)

Gene	Gene name	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon length (bp)
TTR	Transthyretin	F: GTCTGTGCACGT-GTTCAAAAAG R: CCCCTT CTACAAACTTCTCACT	58	119
VCAM1	Adhesion molecule VCAM-1	F: TTGCTAACATTGAGT CCTGTGAGT R: GCTCAGC TCAATTTCTGGATCTCT	64	138
NT5E	5′-Nucleotidase, ecto (CD73)	F: GTATAAGGTGATCCTTC-CGAGCTT R: TTGAGATGTAT TGACAAACCACGC	62	125
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	F: AGACTTACACAAGCTGATC TCACA R: CTGTAAAATCG CCTCCCTGGTAAA	62	119
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	F: AAAACGCCATCGTCCGAA R: AAGTATCACCATCAACCT TGTCC	60	144
MGEA5	Meningioma expressed antigen 5	F: CCGTGTACAAGATTTGCAG AGAAA R: GCTTAAGGAA AGCAGTCCTCCTA	64	110
PPIA[a]	Peptidylprolyl isomerase A (cyclo-philin A)	F: CAACACAAACGGCTCC CAGTT R: CATGGCTTCC ACAATGCTCAT	64	112
GAPDH	Glyceraldehyde-3-phosphate dehy-drogenase	F: GCTTTTAACTCTGGCAAAG TGG R: GGGTGGAATCATA CTGGAACAT	58	94
YWHAZ[b]	Tyrosine 3/tryptophan 5 -monooxy-genase activation protein, zeta polypeptide	F: GGTCTGGCCCTTAACTTCT CTGTGTTCTA R: GCGTGC TGTCTTTGTATGATTCTT CACTT	64	142

Gradient qPCR was used to determine optimal annealing temperatures

[a] Modified from Rai et al. (2010)

[b] PPIA From Seol et al. (2011)

data beyond the single gene approach, and the potential to detect larger-scale systemic changes in biological processes or function in our study populations.

Results

Population dynamics

In this cycle, the first year of the increase phase occurred in 1995. The hare population rapidly increased to a peak in 1998, started declining over the winter of 1999, and reached a low in 2001 (Krebs et al. 2014). From the population peak to the second year of decline, spring density dropped 89 % on control monitoring grids [1998 = 1.93 hares per ha 95 % CI (1.69–2.28), 2000 = 0.21 hares per ha, 95 % CI (0.17–0.35); Fig. 1]. During this same time period, overwinter loss rates increased from 6 to 81 %, and lynx:hare ratios increased 12-fold (Fig. 2). In combination, these data indicate that predation risk increased significantly from the peak to the decline phase of the cycle.

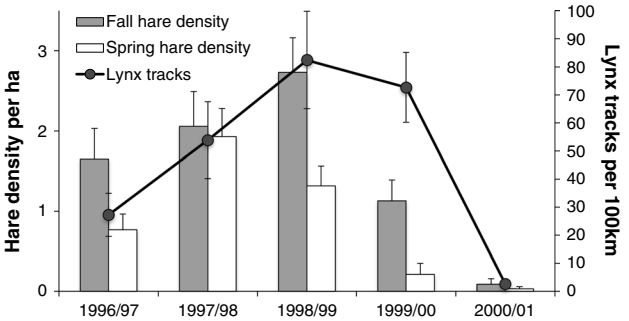

Fig. 1 Fall and spring snowshoe hare (*Lepus americanus*) population densities, and an index of lynx (*Lynx canadensis*) density from winter snow tracking at Kluane Lake, Yukon Territory (1996–2001). Estimates with 95 % confidence limits are shown

Males selected for the microarray and/or qPCR analyses did not significantly differ between years in measures of weight, right hind foot length, or hematocrit (packed

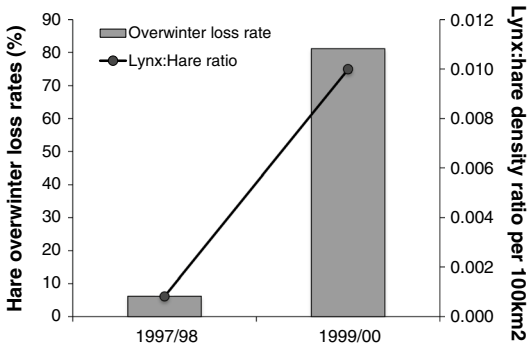

Fig. 2 Overwinter loss rates for snowshoe hares in peak (1997/1998) and decline (1999/2000) years are calculated from fall and spring population censuses. Lynx:hare ratios calculated from mean lynx densities (derived from winter track counts; see Sheriff et al. 2011) and hare spring densities

red blood cell volume), though the general trends for size and hematocrit appear to reverse when sample sizes were increased to include all males trapped during February live-trapping sessions (1998 $N = 25$, 2000 $N = 19$) (Table 2). In the latter case, peak phase animals were slightly heavier and had higher hematocrit indices of condition, though neither difference was statistically significant.

Microarray fold change differences in transcript abundance

After normalization and background correction of the arrays, a total of 23,291 probes showed measurable expression on the arrays. Of these, 644 probes were differentially regulated between peak and decline phase males (independent sample t tests, $p < 0.05$). To increase the power of our analyses, only genes with multiple significant probes were retained, leaving 106 predicted genes, 75 of which have rabbit/homologue Ensembl gene IDs (Online Resource 1). The magnitude of mean absolute fold change (FC) expression differences between years for these genes ranged from 1.06 to 5.91, with the largest fold-change difference in transthyretin (TTR), a thyroid hormone transport gene that was down-regulated in decline phase hares (Table 3).

qPCR validation for candidate genes

Significant differences in microarray transcript abundance in decline ($n = 11$) relative to peak phase males ($n = 10$) were validated with quantitative real-time polymerase chain reaction (qPCR) for six genes. qPCR confirmed the direction of mean microarray fold change regulation for all 6 genes tested, and statistically significant differences in transcript abundance between years were found for 4/6 genes (NAALAD2, NT5E, MGEA5, ATP2A2 $p < 0.03$; TTR $p = 0.066$, VCAM1 $p = 0.34$; Online Resource 2).

Functional enrichment analysis of gene sets

The genes with differential microarray transcript abundance were enriched for six functional annotation clusters (Table 4). Relative to peak phase individuals, hares exposed to higher levels of predation risk during the decline phase showed a significant increase in the expression of gene-sets involved in extracellular processes (maximum Enrichment Score = 2.28), hormone response (ES = 1.77), protein assembly (ES = 1.67), and nucleotide biosynthesis (ES = 1.66). Decline hares also showed a significant down regulation of genes involved in white blood cell activation (ES = 1.81) and red blood cell formation/immune system development (ES = 1.44). Similar functional annotation clusters were obtained whether using human, rat, or mouse sequences, though the significance of individual cluster enrichment varied (Online Resource 3).

Discussion

To our knowledge, this study is the first to link changes in demography to changes in gene expression profiles in a natural population. In the 2-year period spanning the snowshoe hare population peak in 1998 to the second year of the decline in 2000 in the southwestern Yukon, there were marked increases in absolute predator density, lynx:hare ratios, and overwinter loss rates for hares. We identified 106 individual genes whose expression varied as a function

Table 2 Summary of the basic demographic and condition data for all males assessed during february live-trapping sessions, and those used for microarray and qPCR analysis

Values are presented as means ± 1 standard deviation. *P* values from standard *t* tests for differences between years are presented for both groups

	Weight (g)	Right hind foot length (mm)	Hematocrit (%)
All trapped males			
Peak (1998, $n = 25$)	1478.4 ± 160.0	136.4 ± 6.5	44.2 ± 2.9
Decline (2000, $n = 19$)	1439.0 ± 144.7	136.8 ± 5.0	43.3 ± 4.4
p value	0.40	0.80	0.40
Microarray/qPCR males			
Peak (1998, $n = 10$)	1416.6 ± 88.6	135.9 ± 5.5	44.6 ± 3.4
Decline (2000, $n = 11$)	1438.1 ± 95.6	135.6 ± 4.5	45.3 ± 3.0
p value	0.63	0.92	0.70

Table 3 Top 20 genes (characterized or predicted) with significantly different hippocampal RNA transcript abundance on the microarray (after filtering for genes with multiple significant probes only)

Gene symbol	Gene	GO biological process or function	Ensembl gene ID	Probe count	Regulation	Mean absolute fold change	SE
TTR	Transthyretin	Carrier protein; thyroid hormone transport	ENSOCUG00000017348	5	Down	5.91	0.02
AK7	Predicted: adenylate kinase 7-like	Maintaining ciliary structure and function; ATP binding; kinase activity	ENSOCUG00000011516	2	Down	2.62	0.29
CAPSL	Calcyphosine-like	Calcium ion binding	ENSOCUG00000011410	4	Down	2.42	0.18
AKAP14	Predicted: A-kinase anchor protein 14-like	Binds to type II regulatory subunits of protein kinase A and anchors/targets them; spermatogenesis	ENSOCUG00000009612	2	Down	2.22	0.24
WFDC2	WAP four-disulfide core domain protein 2 precursor	Protease inhibitor	ENSOCUG00000009529	2	Up	2.02	0.22
CD1B	T-cell surface glycoprotein CD1b	Immune response; antigen processing and presentation	ENSOCUG00000011270	2	Down	2.01	0.19
DSCC1	Predicted: defective in sister chromatid cohesion 1	DNA replication; post-translational protein acetylation	ENSOCUG00000017952	3	Down	1.84	0.09
OGN	Mimecan	Bone formation; growth factor activity	ENSOCUG00000009017	4	Up	1.81	0.11
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	Peptidase activity; proteolysis	ENSOCUG00000014137	3	Up	1.77	0.17
MGP	Matrix Gla protein	Inhibition of bone formation; Cell differentiation	ENSOCUG00000016964	7	Up	1.54	0.01
CLMN	Predicted: calmin	Actin binding; negative regulation of cell proliferation	ENSOCUG00000010870	2	Up	1.52	0.04
TAC1	Protachykinin-1 preproprotein	Neuropeptide; neurotransmitter; vasodilation; smooth muscle contraction	ENSOCUG00000006182	7	Down	1.50	0.01
YAE1D1	Predicted: hypothetical protein LOC100353779	None available	ENSOCUG00000011496	3	Up	1.49	0.01
RLA-DMB	Histocompatibility antigen DM heterodimer light chain-like precursor	Immune response; antigen processing	ENSOCUG00000026567	5	Up	1.49	0.02
ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	Catalyzes the hydrolysis of ATP; Calcium transport; Ion transport	ENSOCUG00000002688	3	Up	1.44	0.02
NT5E	5′-Nucleotidase, ecto (CD73)	Metal ion binding; nucleotide binding	ENSOCUG00000008919	2	Up	1.44	0.01
LRRIQ1	Predicted: leucine-rich repeats and IQ motif containing 1	None available	ENSOCUG00000017527	2	Down	1.42	0.17
LOC100347507	Predicted: myeloid leukemia factor 1-like	(MLF1) Cell cycle arrest; myeloid progenitor cell differentiation	ENSOCUG00000013549	3	Down	1.42	0.04
COL1A2	Collagen alpha-2(I) chain	Extracellular matrix constituent	ENSOCUG00000012264	3	Up	1.35	0.01
COQ2	Predicted: para-hydroxybenzoate-polyprenyltransferase mitochondrial	Metabolic process	ENSOCUG00000011119	2	Down	1.35	0.13

Gene names and Ensembl IDs are from BetterBunny v.2.1. Gene Ontology for biological processes and functions derived from UniProt using rabbit and orthologous species annotation. Gene regulation corresponds to mean fold change differences in transcript abundance for decline (2000) relative to peak phase (1998) snowshoe hares

Table 4 Significantly enriched (Enrichment Score >1.3) annotation term clusters for genes showing differential microarray transcript abundance

Functional annotation cluster	Annotation terms	Genes	Ensembl gene IDs
(a) Extracellular	GO:0044421 ~ extracellular region part	Apolipoprotein A-II	ENSMUSG00000005681
Enrichment Score: 2.28	GO:0005576 ~ extracellular region	Coagulation factor C homolog (Limulus polyphemus)	ENSMUSG00000020953
(*Mus musculus*)	Secreted	Collagen, type I, alpha 2	ENSMUSG00000029661
		Laminin B1 subunit 1	ENSMUSG00000002900
		Matrilin 2	ENSMUSG00000022324
		Matrix Gla protein	ENSMUSG00000030218
		Osteoglycin	ENSMUSG00000021390
		Similar to signal peptide, CUB domain, EGF-like 2; signal peptide, CUB domain, EGF-like 2	ENSMUSG00000007279 ENSMUSG00000056370
		Surfactant associated protein B	
(b) Hormone response	GO:0009725 ~ response to hormone stimulus	A kinase (PRKA) anchor protein 1	ENSRNOG00000002373
Enrichment Score: 1.77	GO:0048545 ~ response to steroid hormone stimulus	Apolipoprotein A-II	ENSRNOG00000003500
(*Rattus norvegicus*)	GO:0009719 ~ response to endogenous stimulus	Matrix Gla protein	ENSRNOG00000005695
	GO:0010033 ~ response to organic substance	Meningioma expressed antigen 5 (hyaluronidase)	ENSRNOG00000017822
		Neurofilament, light polypeptide	ENSRNOG00000013658
(c) Protein assembly	GO:0065003 ~ macromolecular complex assembly	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	ENSRNOG00000003359
Enrichment Score: 1.67	GO:0070271 ~ protein complex biogenesis		ENSRNOG00000003500
(*Rattus norvegicus*)	GO:0006461 ~ protein complex assembly	Apolipoprotein A-II	ENSRNOG00000003746
		Gap junction protein, beta 1	ENSRNOG00000005695
		Matrix Gla protein	ENSRNOG00000013658
		Neurofilament, light polypeptide	
(d) Nucleotide biosynthesis	GO:0009150 ~ purine ribonucleotide metabolic process	5′ nucleotidase, ecto	ENSMUSG00000032420
Enrichment score: 1.66	GO:0009259 ~ ribonucleotide metabolic process	ATPase, Ca ++ transporting, cardiac muscle, fast twitch 1	ENSMUSG00000030730
(*Mus musculus*)	GO:0006164 ~ purine nucleotide biosynthetic process	predicted gene 10250; hypothetical protein LOC676483; predicted gene 5051; ATP synthase, H + transporting, mitochondrial F0 complex, subunit d	ENSMUSG00000034566
	GO:0009165 ~ nucleotide biosynthetic process		
	GO:0034404 ~ nucleobase, nucleoside and nucleotide biosynthetic process	Predicted gene 4953; similar to ATP synthase, H + transporting, mitochondrial F0 complex, subunit d	
	GO:0034654 ~ nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process		
(e) Regulation of immune activation	GO:0051249 ~ regulation of lymphocyte activation	Inhibin, beta A	ENSG00000122641
	GO:0002694 ~ regulation of leukocyte activation	Tachykinin, precursor 1	ENSG00000006128
Enrichment Score: 1.81	GO:0050865 ~ regulation of cell activation	Vascular cell adhesion molecule 1	ENSG00000162692
(*Homo sapiens*)			
(f) Hemopoeisis and immune development	*GO:0030097 ~ hemopoiesis*	Inhibin, beta A	ENSG00000122641
	GO:0048534 ~ hemopoietic or lymphoid organ development	Myeloid leukemia factor 1	ENSG00000178053
Enrichment Score: 1.44		Vascular cell adhesion molecule 1	ENSG00000162692
(*Homo sapiens*)	*GO:0002520 ~ immune system development*		

Relative to animals from the peak phase, clusters (a) to (d) are up-regulated in decline phase hares, and clusters (e) and (f) are down-regulated. Clusters were generated using homologous human, rat, and mouse protein sequences in DAVID (Huang et al. 2008). Where common clusters were obtained between species, the cluster with the highest Enrichment Score is presented

of predation risk in each of those years. Functional analysis of these genes detected changes in the regulation of six biological pathways involved in nucleotide biosynthesis, protein assembly, immunity, and hormone response. These large-scale functional changes are consistent with physiological and condition evidence that have been previously found for chronic stress-mediated effects of predation during the decline phase of the cycle.

While samples sizes used for our microarray analysis ($n = 8$ per group) appear modest by ecological standards when considering other metrics, they are typical from the standpoint of similar heterologous array studies using non-model species ($n = 2$–12 per treatment group; e.g., Rinaudo and Gerin 2004; Saetre et al. 2004; Brodsky et al. 2005) and even using model species in biomedical contexts (e.g., McGowan et al. 2011; Suderman et al. 2012). Thus, we are confident that sample sizes used were adequate to provide a robust test of our research question. A potential caveat associated with the use of heterologous microarrays is the concern that differences in hybridization intensity may be the result of sequence divergence between the European rabbit (for which the microarray was developed) and the snowshoe hare, rather than true differences in transcript abundance between experimental groups (Buckley 2007). In general, however, studies comparing cross-species gene expression show that though the ability to detect differential hybridization decreases with phylogenetic distance, the magnitude of decrease is relatively moderate (reviewed in Buckley 2007; Kassahn 2008). Eddy and Storey (2002) predicted that heterologous cDNA hybridizations using two mammal species can expect up to 85–90 % cross-reactivity of genes on the array. Renn et al. (2004) compared the gene expression profiles of a variety of fish species hybridized to an African cichlid array and found very high concordance for closely related taxa [<10 million years ago (MYA) divergence], and consistent hybridization results for species diverging up to 65 MYA, though phylogenetic distance decreased the detection of subtly regulated (low fold change) genes. Given that rabbits and snowshoe hares diverged approximately 11.8 MYA, in evolutionary terms, they are relatively closely related by comparison (Matthee et al. 2004). Our successful qPCR validation of microarray findings and functional enrichment results that are consistent with those predicted suggest that the homology between these two species is sufficient to obtain meaningful results, particularly at the level of gene network and biological system interactions. Nonetheless, direct comparison of the rabbit and the snowshoe hare genome, which has recently been sequenced (L. Andersson, personal communication), would allow for more precise estimates of array binding efficiency in the future. This information will also enable more complex functional analyses by including genes in addition to those with known homologies between model species and the rabbit genome.

We expect the stressor of intense predation risk to affect large numbers of genes simultaneously. The functional enrichment analysis of up- and down-regulated gene sets thus provides power that individual gene-level analysis cannot, and an ability to detect larger-scale patterns of regulation between phases of the cycle. In our study, clusters of genes involved in white blood cell activation, immune development, and hemopoeisis (red blood cell formation) showed lower levels of expression in decline phase males. The genes contributing to these clusters were inhibin beta A (INHBA), myeloid leukemia factor 1 (MLF1), tachykinin precursor 1 (TAC1), and vascular cell adhesion molecule 1 (VCAM1). Of note, VCAM1 is a member of the immunoglobulin family that mediates lymphocyte maturation and function, and is critical for resistance to parasitic and viral infection (Deckert et al. 2003; Ou et al. 2008). These results are in line with evidence that hares exposed to high levels of predation risk during the decline show signs of chronic stress and are in poor condition. They have compromised immune systems (lower levels of lymphocytes, neutrophils, and monocytes), and reduced hematocrit values, a commonly used integrative index of condition (Boonstra and Singleton 1993; Boonstra et al. 1998b; Breuner et al. 2013).

The list of genes showing increased transcript abundance in males from the decline phase was also significantly enriched for annotation terms involved in steroid hormone response. The genes contributing to this cluster (a kinase anchor protein 1, apolipoprotein A-II, matrix Gla protein, meningioma expressed antigen 5, and neurofilament light polypeptide) represent 16 % of the total input gene list. This cluster highlights a potential upstream regulatory mechanism for chronic stress-induced increases in HPA axis reactivity seen during the decline phase, when hares have heightened HPA axis responses to a stressor, with greater plasma cortisol and glucose levels both at capture and in response to a hormonal challenge (Boonstra et al. 1998b).

Of the 106 genes that showed significant differences in microarray transcript abundance between phases, the largest difference was in transthyretin (TTR), a gene expressed almost six times more in peak than decline phase males. Transthyretin is mainly synthesized in the liver and the choroid plexus, and is involved in the transport of thyroid hormones and retinol binding protein (Schreiber and Richardson 1997). TTR has a glucocorticoid responsive element in the 3' region of the first intron that is conserved among mice, humans, and rats (Wakasugi et al. 1986; Sasaki et al. 1985; Martinho et al. 2012). A number of laboratory studies have found evidence for stress-induced regulation of TTR expression. Martinho et al. (2012) found that rats that

experienced the psychosocial stresses of both short- and long-term increased housing density had increased TTR expression in the choroid plexus, liver, and cerebrospinal fluid. In contrast, TTR mRNA expression decreases in the brain cortex of mice subjected to repeated immobilization, a form of chronic physical and psychological stress (Joo et al. 2009). In addition, a microarray study by Kohda et al. (2006) found that juvenile rats subjected to maternal separation early in life show lower levels of hippocampal TTR when tested at 13 weeks of age. While our qPCR analysis also found increased transcript abundance of TTR in peak relative to decline phase males, this result bordered on statistical significance ($p = 0.066$). This inconsistency may stem from erroneous probe annotation, or from microarray and qPCR probe sets' recognition of alternative transcript splice variants. Nonetheless, taken together, these results suggest a potential role for transthyretin in stress susceptibility. It is also possible that indirect predator-induced changes in hare foraging behavior and diet composition during the decline phase might influence the expression of TTR (Straus et al. 1994). In either case, further work is required to understand how and when this might occur.

The mechanisms underlying the snowshoe hare population cycle are an area of active investigation. In addition to physiological impacts on individuals directly exposed to predation risk, predator-induced maternal effects may also play a role in this species. If all individuals are similarly affected, there is the potential for entire populations to be transformed. Females exposed to a simulated predator during pregnancy have elevated fecal cortisol metabolite concentrations (a non-invasive index of circulating stress hormones) at birth (Sheriff et al. 2009). This predator-induced stress affects reproductive output, with stressed females giving birth to fewer, smaller, and lighter offspring than control mothers (Sheriff et al. 2009). In addition, females with high fecal cortisol metabolite levels produce offspring with increased reactivity of the stress axis at weaning (Sheriff et al. 2010). Maternal programming of the stress axis may be mediated by a variety of factors, including circulating stress hormones in utero (Seckl 2004; Matthews et al. 2004), maternal nutrition (Sasaki et al. 2013), and postnatal maternal care (Szyf et al. 2005; McGowan et al. 2011). If predator-induced alterations in offspring morphology, physiology, and behavior lead to increased fitness and survival in a high-predation environment, such as that seen during the decline phase of the cycle, this maternal programming of offspring may effectively be preparatory and adaptive rather than an inevitable negative consequence of predator stress (Love and Williams 2008; Sheriff and Love 2013).

In summary, we found heterologous microarray technology to be a useful tool for detecting changes in gene expression in wild snowshoe hares, yielding valuable insights into biological system interactions. Our results suggest that there are pronounced transcriptional changes in the brains of hares correlated with the risk of predation during different phases of the population cycle, and that these changes are concordant with the physiological effects predicted by the Chronic Stress Hypothesis of predation. Much can be gained from applying genomic investigations to wild populations for which rich natural history knowledge is available, and it is only by studying the mechanisms underlying phenotypic variation in nature that we can determine whether they have any adaptive significance (Travers et al. 2007). Variation in predator-induced stress strongly impacts hare physiology and reproduction, and we now have evidence that suggests these effects are accompanied by changes in gene expression patterns. We do not yet know whether these changes in expression are driving the alterations in physiology, or vice versa. Future work must resolve the molecular mechanisms underlying this differential transcription in the hippocampus. Studies of epigenetic patterns and programming will be key to determining whether elevated predation risk can lead to fundamental changes at the level of DNA, whether these stress-induced changes are permanent or reversible, to what extent they can be inherited by offspring, and ultimately, how they can affect hare population dynamics and the 10-year cycle.

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